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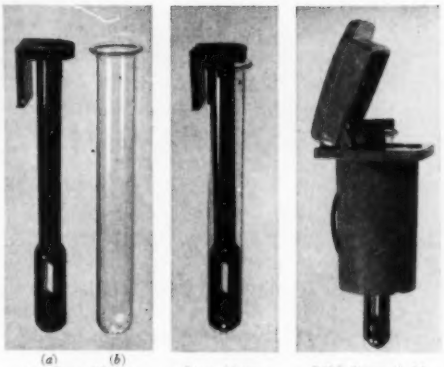

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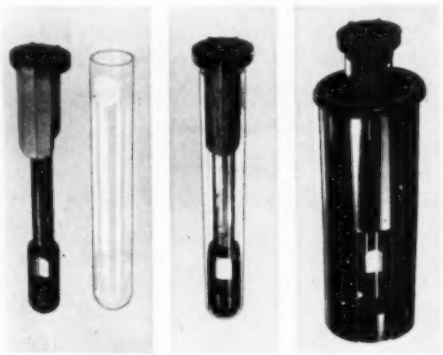

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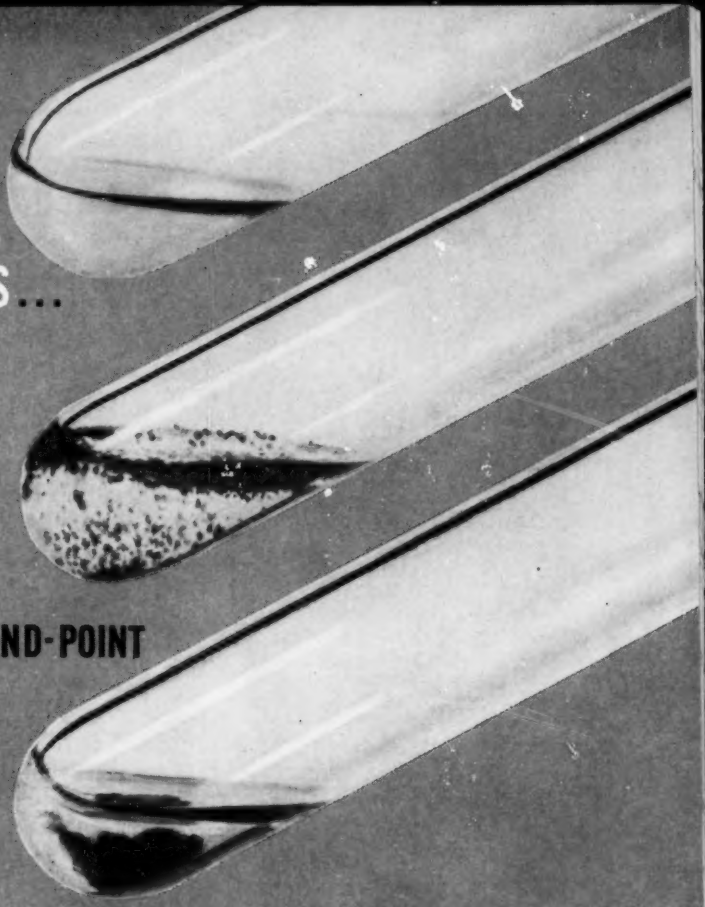

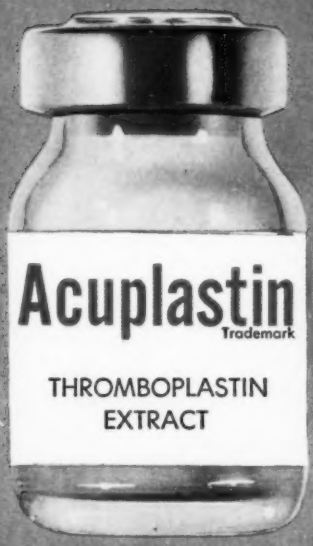

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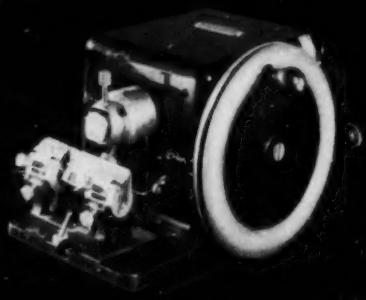

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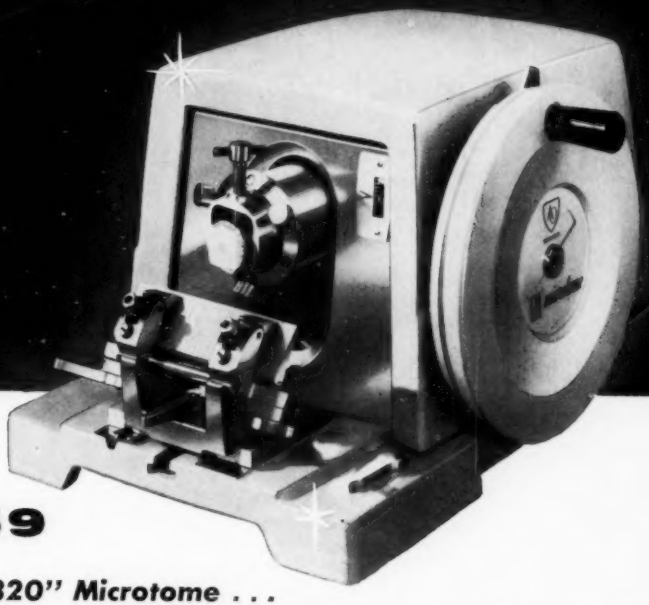

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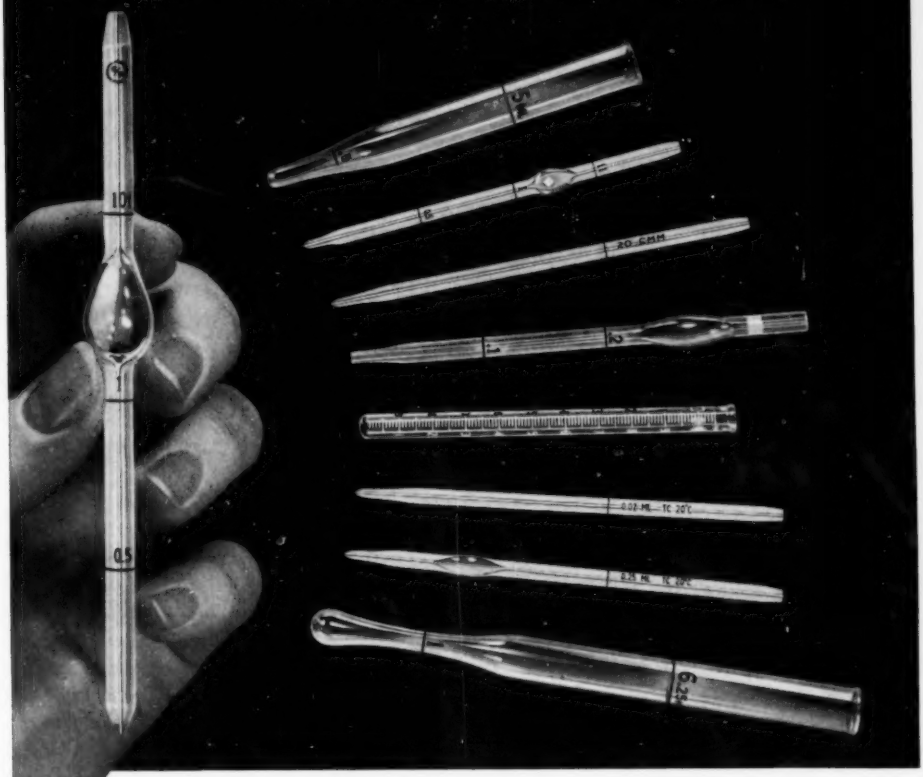

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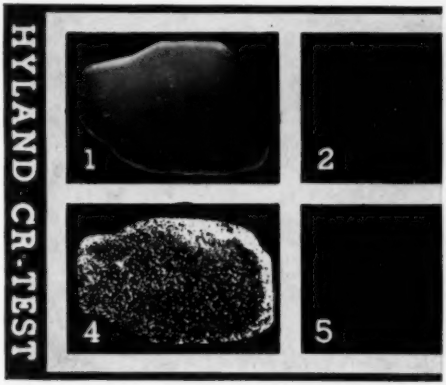

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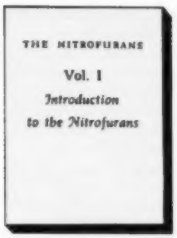

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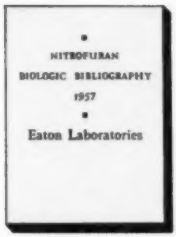

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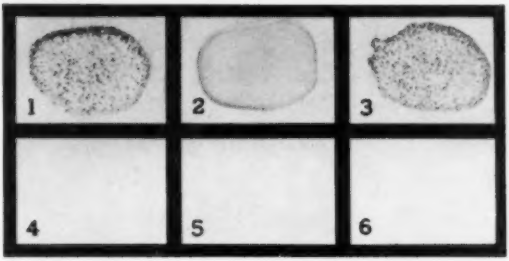

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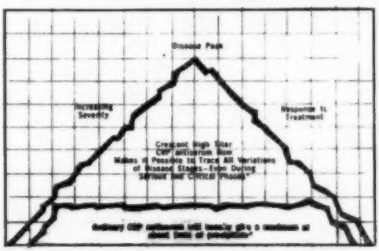

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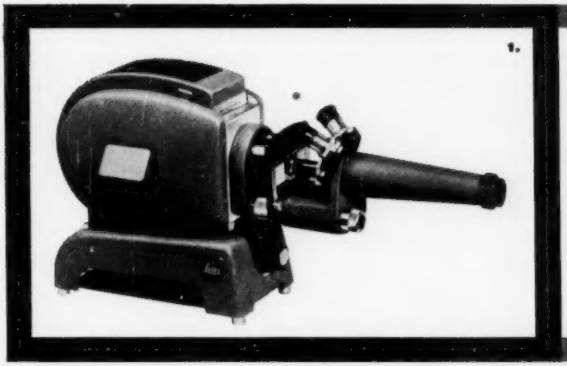

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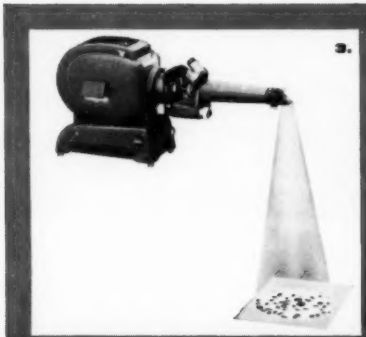

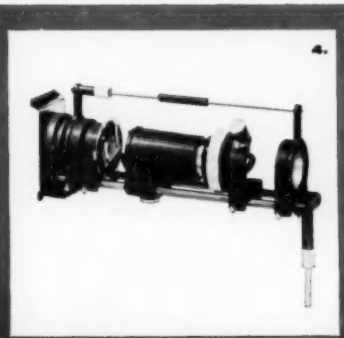

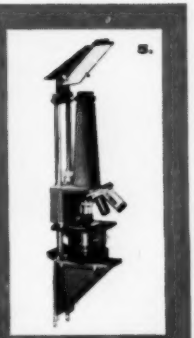

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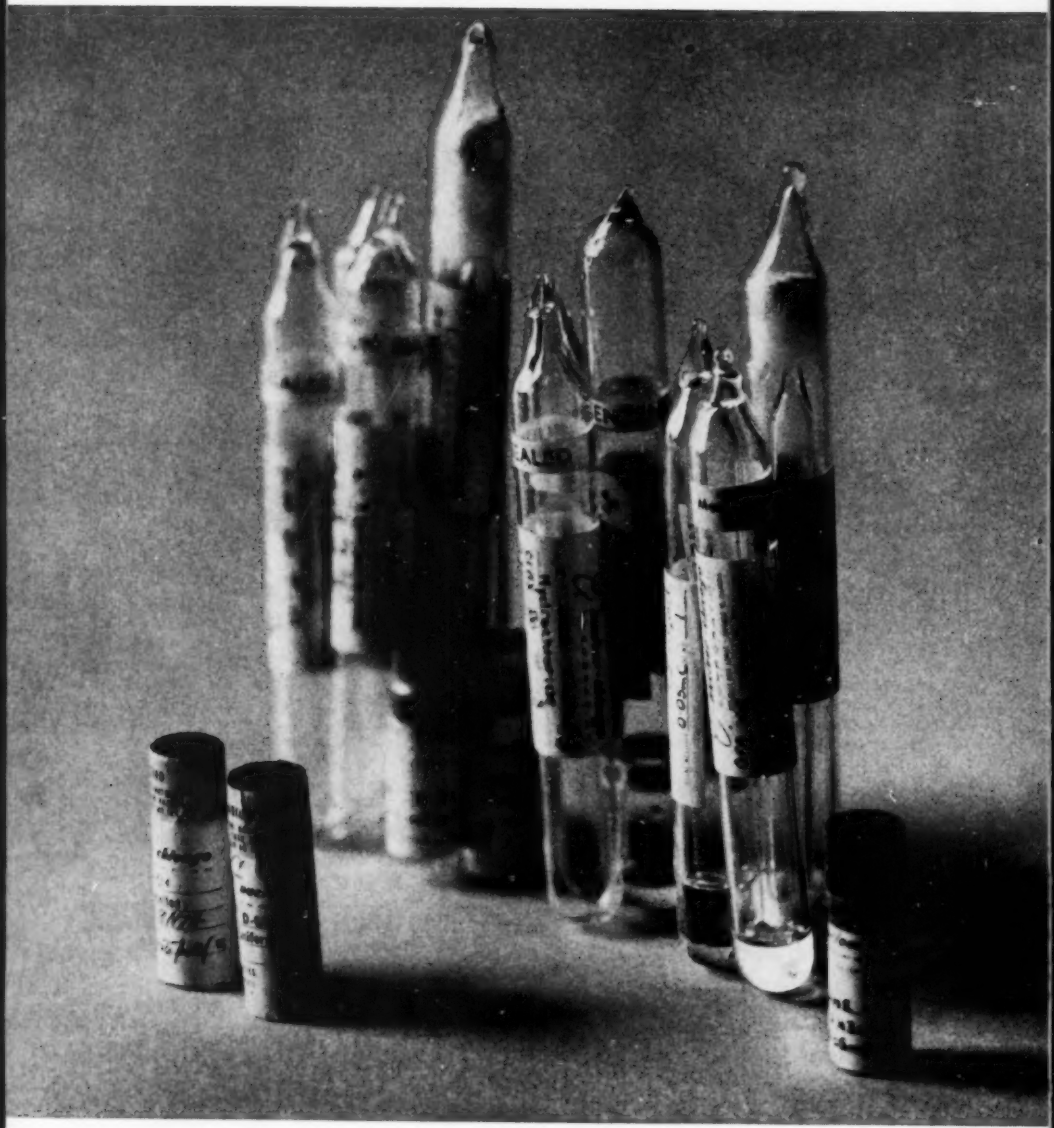

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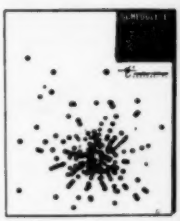

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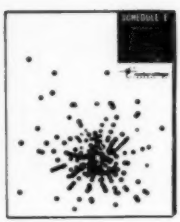

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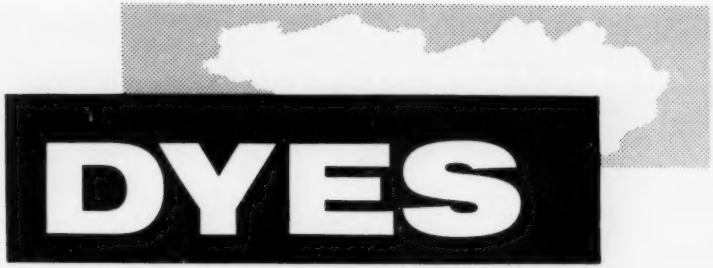

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THE EFFECT OF HEPARIN ON PLASMA THROMBOPLASTIN FORMATION

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BIGGS, Douglas, and MacFarlane[1] were the first to show that heparin inhibited the formation of plasma thromboplastic activity in vitro, thereby interfering with the conversion of prothrombin to thrombin. This was subsequently confirmed by MacMillan and Brown[2] as well as Douglas.[3] Until recently,[4] no definite explanation for this inhibition had been offered.

The following are the results of some of our studies on the effect of heparin on thromboplastic activity as produced in the thromboplastin generation test of Biggs and Douglas,[5] as well as on the various components of the generation mixture.

METHODS AND MATERIALS

Collection of blood samples and performance of the thromboplastin generation test were as previously described.[6]

Heparin.—Heparin sodium* 1,000 U.S.P. units (10 mg.) per milliliter.

Protamine.—Protamine sulfate powder.†

Lecithin.—Inositol phospholipid concentrate‡ from soya lecithin.

All solutions of heparin, protamine, and lecithin were made in 0.85 per cent saline.

From the Departments of Pathology, Harvard Medical School, Boston, and the Veterans Administration Hospital, West Roxbury, Mass.

This investigation was aided in part by United States Public Health Service Grant H-2963.

The results of this study were reported, in part, at the seventh Congress of the International Society of Blood Transfusion, Rome, Sept. 3-6, 1958.

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*The Upjohn Company, Kalamazoo, Mich.

†Delta Chemical Works, Inc., New York, N. Y.

‡Associated Concentrates, Woodside, Long Island, N. Y.

RESULTS

Various concentrations of heparin in saline solution were added to the mixture prior to the inception of thromboplastin generation, i.e., before the addition of calcium, or after 3 minutes of incubation, that is, at the time of so-called "optimum" thromboplastic activity (Fig. 1). When added prior to the inception of thromboplastin generation, heparin markedly delayed or inhibited the rate at which the thromboplastic activity developed. However, contrary to what has been reported previously by Biggs and associates,[1] as well as MacMillan and Brown,[2] it was found that on adding the heparin to the mixture after 3 minutes of incubation, i.e., at the point where full thromboplastic activity is reached, there was a marked and rapid deterioration of activity. This effect was usually greater than the inhibition produced by adding the same concentration of heparin to the mixture prior to the onset of generation.

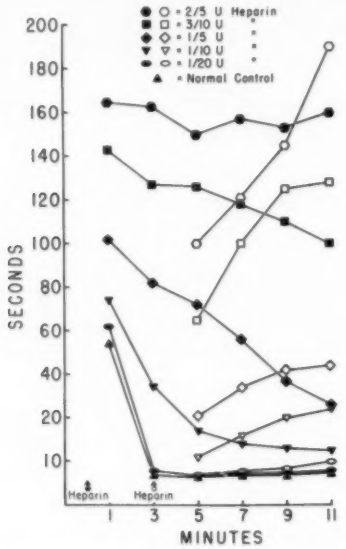

Fig. 1.—The effect of heparin on thromboplastin generation. Generation mixture was composed of: 0.2 ml. of frozen and thawed suspension of platelets in saline, 0.2 ml. of a 1:10 dilution of serum in saline, 0.2 ml. of a 1:5 dilution of $BaSO_4$-adsorbed, oxalated plasma in saline, and 0.2 ml. 0.02 M calcium chloride (total volume 0.8 ml.). Various concentrations of heparin (0.01 ml.) were added to the mixture before the addition of calcium or 3 minutes after the addition of calcium.

The prolonged clotting times produced were not due to heparin being transferred to the substrate. If the same amounts of heparin were added to each substrate tube, instead of being added to the generation mixture, no change in the normal curve of thromboplastin formation occurred. Actually, in the test, only one-eighth of this amount would be carried over from the mixture.

As has been demonstrated by van Creveld and Paulssen,[7] Deutsch and coworkers,[8] Garrett and Klein,[9] and Burstein and Guinand,[10] platelets themselves

contain an antiheparin factor, platelet factor IV. Therefore, a parallel experiment was performed in which a human brain cephalin, prepared according to the method of Bell and Alton,[11] was substituted for the platelets in the generation mixture (Fig. 2). The curves obtained are similar to those when platelets were present. Here, however, the amount of heparin needed to produce an equivalent effect is much less since cephalin does not have an antiheparin activity. Similar results were obtained when a soybean lecithin was used as the source of platelet factor III in the generation mixture.

Both Conley and his associates[12] as well as Gross and his co-workers[13] have shown that a decreased number of platelets enhances the action of heparin in delaying coagulation of plasma. Likewise, decreasing the number of platelets in the generation mixture increases the inhibitory effect of heparin. If the platelets are deleted from the generation mixture, being replaced by an equal volume of saline, a significant amount of thromboplastic activity is developed although

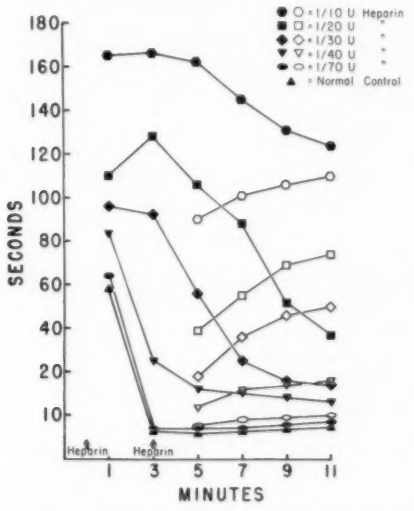

Fig. 2.—The effect of heparin on thromboplastin generation. Components of the mixture were the same as in Fig. 1 except for the substitution of 0.2 ml. of cephalin for the platelet suspension.

both the rate and amount are decreased (Fig. 3). Adding as little as 0.05 unit of heparin (0.5 gamma) to this mixture greatly inhibits the formation of thromboplastin. It may be noted that the smallest concentration of heparin required to affect thromboplastin generation is the same for those mixtures in which platelets are deleted or in which cephalin is substituted for platelets; that is, in those mixtures in which the platelet antiheparin factor is absent.

When platelets are used in the generation mixture, doubling the concentration of platelets tends to counteract the heparin effect. This is probably due to the increase in the concentration of the platelet antiheparin factor (Fig. 4).

However, with a fourfold concentration of platelets, the correction of thromboplastin generation is not as great, possibly due to the superimposed antithromboplastic effect of excessive platelets.[14]

This inability of the platelet thromboplastic factor (platelet factor III) to counteract heparin is more clearly demonstrated when human brain cephalin is

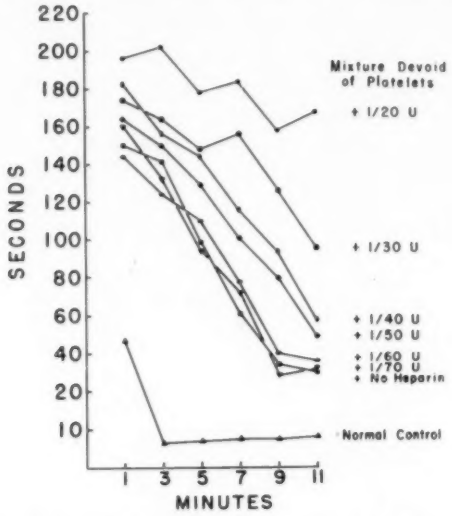

Fig. 3.—The effect of heparin on a thromboplastin generation mixture devoid of platelets. The heparin was added to the mixture before the addition of the calcium.

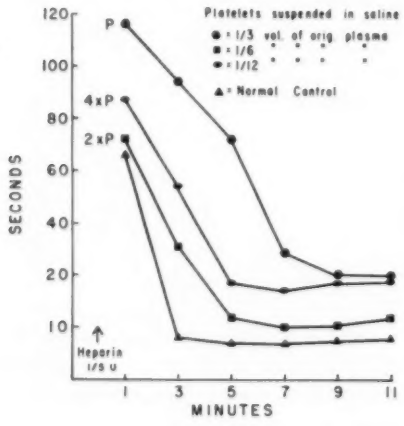

Fig. 4.

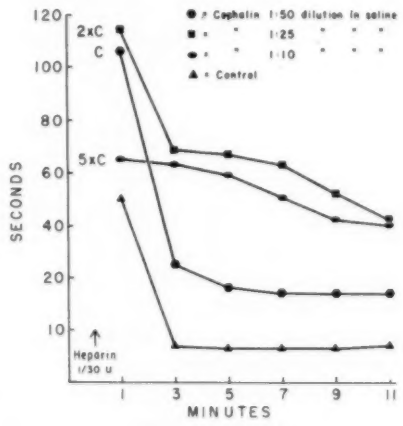

Fig. 5.

Fig. 4.—The effect of increased concentrations of platelets on the heparin inhibition of thromboplastin generation.

Fig. 5.—The effect of increased concentrations of cephalin on heparin inhibition of thromboplastin generation.

substituted for platelets (Fig. 5). Increasing the concentration of this constituent progressively inhibits the generation of thromboplastin. Similar curves were obtained when soybean lecithin was used as a source of platelet factor III.

If the serum is omitted from a generation mixture containing platelets, a lesser amount of thromboplastic activity is generated than when either the adsorbed plasma or platelets are omitted (Fig. 6). The addition of heparin to such a mixture devoid of serum produces no significant change in the curve of thromboplastin generation. Adding heparin to a mixture devoid of adsorbed plasma produces a defect in thromboplastin generation almost equal to that of serum deletion alone. In each instance where a component of the generation mixture was omitted, it was replaced by an equal volume of saline. If cephalin were substituted for the platelets and 0.05 unit of heparin added to the mixtures, no significant difference in the curves was noted.

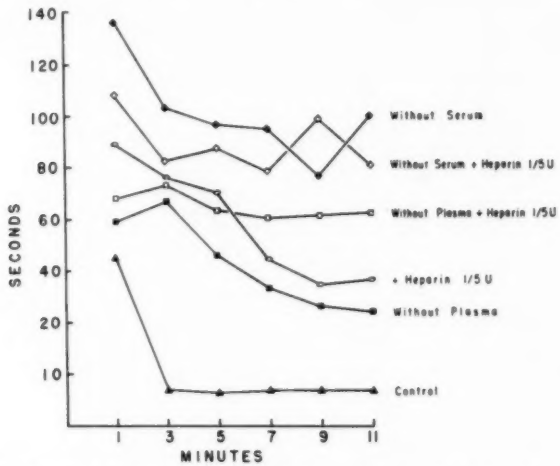

Fig. 6.—The effect of heparin on thromboplastin generation mixtures devoid of either serum or adsorbed plasma.

We then studied the effect of varying the concentrations of the serum or adsorbed plasma on the heparin inhibition of thromboplastin generation (Fig. 7). The concentration of the platelet suspension was kept constant. Increasing the concentration of the serum counteracted the inhibitory effect of heparin. Increasing the concentration of the adsorbed plasma had only a slight tendency to correct the heparin inhibition. If, however, human brain cephalin were substituted for the platelets in the incubation mixture, increasing the concentration of the adsorbed plasma only enhanced the inhibitory effect of heparin (Fig. 8). Again, as can be seen, increasing the concentration of the serum counteracted the heparin effect.

If barium sulfate-adsorbed serum were substituted for normal serum in the generation mixture, about the same degree of thromboplastic activity was

obtained as with the mixture in which saline replaced the serum. The addition of heparin to this mixture did not cause further inhibition of generation. Increasing the concentration of the adsorbed serum to full strength did not alter the curves with or without heparin. From this, one might conclude that heparin reacts with one of the factors which is adsorbed by barium sulfate. These include factor VII (stable factor, proconvertin, pro-SPCA), Stuart-Prower factor, and PTC (plasma thromboplastin component, factor IX, Christmas factor).

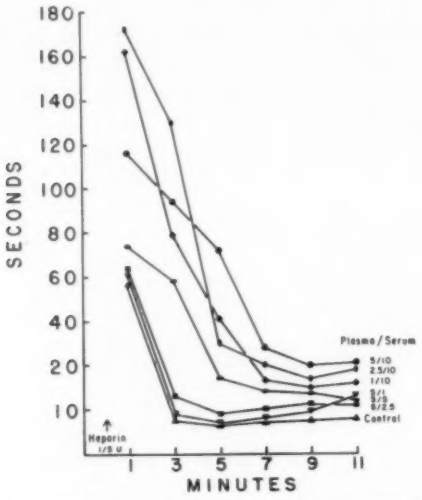

Fig. 7.—The effect of varying concentrations of serum or adsorbed plasma on the heparin inhibition of thromboplastin generation. On the right side of the graph are the reciprocals of the adsorbed plasma and serum dilutions. The generation mixture included a suspension of platelets.

When serum from an individual with a congenital deficiency of factor VII was substituted for the normal serum in the mixture, a normal generation curve was obtained whether platelets or cephalin was used as the source of platelet factor III. The mixture containing factor VII deficient serum reacted in the same way to heparin as the one containing normal serum, and increasing the concentration of this serum in the mixture also counteracted the heparin effect. This was also found to be true when serum from the blood of a severe hemophilic patient was used in the same way.

If serum from an individual with a congenital Stuart-Prower factor deficiency were substituted for the normal serum in the mixture, an abnormal thromboplastin generation was obtained which was further inhibited by heparin. Increasing the concentration of such serum to full strength increased the amount of thromboplastic activity generated. If the same concentration of heparin were added to such a mixture, no decrease in the amount of thromboplastin generated occurred.

If, now, serum from an individual with a congenital PTC or Christmas factor deficiency were substituted for the normal serum, abnormal thromboplastin generation again was obtained which was further inhibited by heparin. Heparin also inhibited thromboplastin generation when full-strength PTC-deficient serum was used. However, this varied according to the severity of PTC deficiency. Addition of normal serum to the PTC-deficient serum (1:9) tended to correct the defect in thromboplastin generation and increasing the concentration of such a combined serum also counteracted the heparin inhibiting effect.

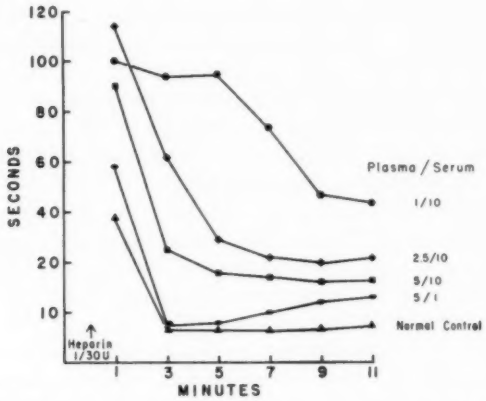

Fig. 8.—The effect of varying concentrations of serum or adsorbed plasma on the heparin inhibition of thromboplastin generation. Cephalin was substituted for platelets in the generation mixture. On the right side of the graph are the reciprocals of the adsorbed plasma and serum dilutions.

The maximum thromboplastic activity generated with these various mixtures is presented in Table I.

TABLE I. MAXIMUM "THROMBOPLASTIN" GENERATED (IN SECONDS)

SERA	GENERATION MIXTURE*	WITHOUT HEPARIN	PLUS 0.2 U. HEPARIN	GENERATION MIXTURE*	WITHOUT HEPARIN	PLUS 0.05 U. HEPARIN
Normal serum	5:10 Platelets	7.5	47.5	5:10 Cephalin	8	27
	5:1 Platelets	7.5	8	5:1 Cephalin	8	9
$BaSO_4$-adsorbed serum	5:10 Platelets	61	78.5	5:10 Cephalin	66	67.5
	5:1 Platelets	67	74	5:1 Cephalin	61	66
Factor VII deficient serum	5:1 Platelets	7	9.5	5:1 Cephalin	7	7
Stuart-Prower factor deficient serum				5:1 Cephalin	28.5	24
Factor IX (PTC) deficient serum	5:1 Platelets	42	68	5:1 Cephalin	39.5	51

*Reciprocals of the plasma:serum dilutions.

DISCUSSION

Soon after the discovery of heparin, Howell[15] stated that it acted as an anticoagulant by inhibiting the conversion of prothrombin to thrombin, although he believed that this was due to a reaction of heparin with prothrombin. Since then, many investigators have claimed that tissue thromboplastin or cephalin is antiheparin[16-18] and, vice versa, that heparin is antithromboplastic,[2, 17, 19-21] this latter property explaining the delayed production of thrombin in vitro.

Quick,[22] however, has averred that tissue thromboplastin does not directly neutralize heparin but antagonizes the anticlotting action of heparin by accelerating the conversion of prothrombin to thrombin which in turn acts against heparin. In doing so, he emphasized that, in heparin studies, the substrate plasma or fibrinogen clotted by thrombin must be prothrombin free, since any contaminating prothrombin would be converted by thromboplastin to thrombin to interfere with heparin. It must be pointed out that aluminum hydroxide, which he used for adsorption of prothrombin, would also adsorb accelerators of tissue thromboplastin such as PTC, factor VII, and Stuart-Prower factor. Indeed, MacMillan and Brown[2] showed that heparin had a deleterious effect only on serum-activated tissue thromboplastin. In the experiments reported by Wadsworth and his associates,[19] rabbit serum was incorporated in all of the clotting mixtures which were used to demonstrate the antiheparin activity of cephalin.

In addition, it has been shown that heparin interferes with the formation of plasma thromboplastin in vitro,[1-4] that is, in the thromboplastin generation test of Biggs and Douglas. We have confirmed these observations but, in contrast to what has been reported, have found that the same concentration of heparin causes marked deterioration of "formed" thromboplastin. We have been unable to explain this discrepancy of findings. Several of these authors[2, 4] have also found that the serum of individuals receiving heparin either intravenously or intramuscularly is defective when substituted for a normal serum in the thromboplastin generation test.

As Miale and Wilson[23] have pointed out, thrombin is evolved in the thromboplastin generation test, probably from a small amount of residual prothrombin in the serum component. Since 1901, the theory of an autocatalytic function of thrombin in blood coagulation has been frequently proposed. The progression of these concepts was reviewed in 1951 by Stefanini[24] who stated that thrombin plays a twofold part in blood coagulation: (1) the aggregation and lysis of platelets necessary for thromboplastin formation, and (2) the activation of an accelerator of prothrombin conversion to thrombin. Quick,[25] in 1953, discounted the "labilizing" effect of thrombin on platelets and postulated that thrombin was responsible for the activation of "thromboplastinogen." More recently, he has suggested that it could be just as likely PTC, or some other thromboplastic factor, which on activation by thrombin initiates the clotting reaction.[26]

Both our own observations, presented here, and those of O'Brien[4] would indicate that heparin interferes with the formation of plasma thromboplastin,

and thus with the conversion of prothrombin to thrombin, by interacting with or antagonizing whatever is responsible for PTC (Christmas factor, factor IX) activity.

In view of these findings, and by interpolation into the scheme of whole blood coagulation, we should like to suggest that the following happens. In the initiation of coagulation a small amount of thrombin is evolved which forms a complex with PTC to produce PTC activity. The active PTC then enters into a reaction with the other thromboplastic factors, as postulated by Bergsagel,[27, 28] to produce plasma thromboplastic activity. This thromboplastin would then, in turn, convert prothrombin to more thrombin setting into motion the autocatalytic process. Such a theory would not exclude the existence, in addition, of a direct accelerator of prothrombin conversion that would also be activated by thrombin.

Therefore, anything which interferes with the formation of this thrombin-PTC complex would cause poor thromboplastin generation, whether it be a lack of thrombin or PTC or an antagonist of either one. It is well known, that in a deficiency of any of the thromboplastic factors, thrombin generation is abnormal.

We believe that heparin inhibits the formation of the thrombin-PTC complex by its antithrombic action whereas protamine does so by "blocking" PTC. It is also possible that heparin may, in addition, act against PTC itself. Weiner and Jiminez[29] have shown a positive correlation, in the recalcification clotting time of plasma, between the sensitivity to heparin and the sensitivity to protamine.

Since the amount of thrombin activity produced in a thromboplastin generation mixture is directly proportional to the concentration of prothrombin in the serum used, in those tests in which the inhibiting effect of heparin was counteracted by a greater concentration of serum, it was probably because of the increased amount of thrombin evolved. Similarly, in previous observations, in which greater concentrations of serum corrected protamine inhibition of thromboplastin generation, it may have been due to the increased concentration of PTC incorporated in the mixture.

Evidence has been presented in the literature that heparin may form physicochemical combinations with various proteins, and that, under certain conditions, these combinations are dissociable, liberating inhibited biologic activities.[20, 30, 31] In a like manner, we have found that the combination of heparin or protamine with one of the components of the thrombin-PTC complex is reversible. The inhibition of thromboplastin generation by either heparin or protamine can be corrected by the addition of the other to the mixture. Thus, heparin and protamine have a greater mutual affinity for one another than that of either one for "PTC."

In all of this, the effect of heparin and protamine on the thrombin-PTC complex would be analogous to that on the thrombin-fibrinogen complex as postulated by Amann and Werle.[31]

That inhibition of thromboplastin production is one of the major points of action in the role of heparin as an anticoagulant is a distinct possibility.

The ability of heparin to interfere with the formation of a thrombin-fibrinogen complex necessary for fibrin formation may act merely as a "backstop" phenomenon. Certainly, heparin has more than one site of action in the coagulation scheme.

It might be more interesting to speculate that since various coumarin and inandione derivatives (with one reported exception[32]) cause a depletion of PTC activity,[32-41] this may be the common denominator of all effective anticoagulant therapy. Soulier[42] has maintained that the heparin tolerance test is a more sensitive index of adequate dicumarolization than the one-stage prothrombin time determination. Verstracte and his co-workers,[43] in a discussion of the heparin tolerance test, pointed out that a deficiency of PTC makes the plasma very sensitive to the action of heparin, much more so than a deficiency of AHG, or fibrinogen, or platelets.

Thus, determinations of PTC-deficiency, whether by the thromboplastin generation test, cross-correction tests, or the heparin tolerance test, may be a helpful adjunct to the determination of an adequate anticoagulant state in the prevention and treatment of thrombosis.

SUMMARY

1. Heparin markedly inhibits the formation of plasma thromboplastic activity as measured in the thromboplastin generation test of Biggs and Douglas.

2. Heparin has a similar effect on "formed thromboplastin" after the point of optimum generation.

3. Heparin, by being antithrombic, may inhibit plasma thromboplastin generation by interfering with the formation of a thrombin-PTC complex necessary for PTC activity.

We wish to thank Drs. John B. Graham, Cecil Hougie, F. Stanley Porter, Frederick S. Bigelow, and Mario Stefanini for samples of various factor-deficient sera used in this study.

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EXPERIMENTAL STUDIES ON BLOOD-SPINAL FLUID BARRIER FOR BILIRUBIN

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WHILE the etiology of kernicterus is incompletely understood, current thought favors the concept that an increased concentration of indirect bilirubin in the serum constitutes an essential element in its development. According to certain authors[1, 2] there exists a good correlation between concentrations of bilirubin in the serum and incidence of kernicterus in full-term newborn infants. Yet, repeated observations reveal a wide individual variation in the susceptibility of the central nervous system to hyperbilirubinemia. The facts suggest that the neonatal period is the most favorable time for the development of kernicterus and, moreover, that a brain lesion may develop at a lower concentration of bilirubin in the serum of prematurely born infants than those born at term.

Other investigators,[3-7] supported by in vitro experiments with homogenized brain tissue, stress the view that a toxic action of bilirubin metabolism may constitute the etiologic basis of kernicterus. The evidence obtained supports the hypothesis that the pathogenesis of kernicterus may be explained in terms of a metabolic injury of the central nervous system caused by bilirubin. The extent to which neonatal jaundice may become critical in the development of such an injury depends on both the actual concentration of bilirubin in the serum and possibly the functional maturity of the blood-brain barrier system in the very young infant.[8-10]

The purpose of this paper is to describe some observations on serum and spinal fluid bilirubin levels after the intravenous injection of bilirubin into rabbits. Four types of studies were carried out. We first attempted to confirm the work of Ernster, Herlin, and Zetterström[9] that the intracisternal injection of p-chloromercuribenzoate will result in damage to the blood-brain barrier and cause an increased permeability for bilirubin. We then proceeded to study the effect of body size upon its permeability. We then investigated the influence of sodium glucuronate, Versenate, Hykinone, and Gantrisin upon the spinal fluid bilirubin levels. Finally, we examined the effect of anoxemia upon the hyperbilirubinemia. It is hoped the data presented will lead to a better understanding of the physiologic mechanisms involved in the transport of bilirubin across the blood-brain barrier.

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*Twenty-two millimoles $NaHCO_3$, 24 mM. NaCl, 0.98 mM. KCl, 0.24 mM. KH_2PO_4, 0.24 mM. $MgSO_4$, and 0.02 M glucose in 1,000 ml. of distilled water.

MATERIALS AND METHODS

Rabbits weighing between 0.5 and 2.0 kilograms were anesthesized by the intravenous injection of the 7 ml. per kilogram body weight dose of 20 per cent urethane solution. The bilirubin was prepared by dissolving 0.56 Gm. of crystalline bilirubin (Pfanstiehl) in 100 ml. of a phosphate-Ringer-bicarbonate buffer.* A dose of 12, 24, 36, or 48 mg. per kilogram body weight was injected intravenously into the rabbit over a 1-minute period. Spinal fluid was obtained by cisternal puncture with the rabbit being placed in the same position as in a human adult. The blood samples were collected by heart puncture. In each experiment, simultaneous blood and spinal fluid samples were obtained 15, 30, and 60 minutes after the injection of bilirubin. None of the animals were used a second time for the study.

The spinal fluid bilirubin was determined by method (a) described by Nasralla, Gawronska, and Hsia.[10] Although this method is the best available, there may be some difficulty in assessing reliability of the results when the bilirubin in the spinal fluid is less than 0.1 mg. per 100 ml. Nasralla and associates had estimated the spinal fluid bilirubin by two methods, and on reviewing their results a surprisingly good correlation was found between the two values below 0.1 mg. per 100 ml. In spite of this, emphasis has not been placed on spinal fluid bilirubin results of less than 0.1 mg. per 100 ml. when interpreting the data. The spinal fluid protein was assayed by a modification of the method of Denis-Ayer.[11] The serum bilirubin levels were determined by a modification of the method of Hsia, Hsia, and Gellis.[12]

RESULTS

1. The Effect of p-Chloromercuribenzoate.—Of 8 rabbits weighing approximately 2 kilograms, 4 were injected intracisternally with 0.1 ml. of 0.5 per cent p-chloromercuribenzoate (PCMB),* and the other 4 with 0.1 ml. physiologic saline solution. Loads of 12, 24, 36, and 48 mg. per kilogram of body weight of bilirubin were injected intravenously immediately after the cisternal injection.

Table I shows the effect of treatment with PCMB intracisternally on the penetration of bilirubin from blood into spinal fluid. It can be seen that in the rabbits treated with PCMB, the ratio of spinal fluid bilirubin to serum bilirubin (SFB/SB) using the 4 dosages of bilirubin are, respectively, 27.2, 35.8, 23.3, and 11.6 times higher than that of the control rabbits at 30 minutes. There was a similar increase in the protein content of the spinal fluid so that the ratio of spinal fluid bilirubin to spinal fluid protein remained unchanged. With the exception of the lowest bilirubin dose, all of the rabbits treated with PCMB expired, while all of the saline treated controls survived.

The increased penetration of bilirubin into the central nervous system, as a consequence of the treatment with PCMB, could be further ascertained by the examination of the brains. A distinct yellow coloration of the brain of the rabbits treated with PCMB, indicating an accumulation of bilirubin, could be observed all over the surface of the brain, especially on the cerebellum and pons but no actual yellow staining was left after the tissues were fixed in formalin solution for a week.

2. Influence on Body Weight.—The rabbits weighing from 0.55 to 2.00 Kg. were given a 48 mg. per kilogram of body weight load of bilirubin intravenously.

*Lot 2209 p-chloromercuribenzoate, sodium (purified form), California Foundation for Biological Research.

TABLE I. EFFECT OF INTRACISTERNALLY INJECTED p-CHLOROMERCURIBENZOATE ON THE CONTENT OF BILIRUBIN AND PROTEIN OF CEREBROSPINAL FLUID IN RABBITS AT VARIOUS TIMES AFTER THE INJECTION OF DIFFERENT AMOUNTS OF BILIRUBIN I.V.

RABBIT NO.	WEIGHT OF RABBIT (KG.)	DOSE OF BILIRUBIN INJECTED (MG./KG.)	TIME (MINUTES)	INTRACISTERNAL TREATMENT	SERUM BILIRUBIN (MG./100 ML.)	CSF† BILIRUBIN (MG./100 ML.)	CSF PROTEIN (MG./100 ML.)	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN	RATIO CSF BILIRUBIN TO CSF PROTEIN
1	1.64	12	15	PCMB*	4.5	0.62	547.2	0.1378	0.0011
			30		2.5	0.87	754.5	0.3480	0.0011
			60		0.3	1.00	1670.4	3.3333	0.0005
2	1.93	12	15	Saline	6.5	0.02	17.5	0.0030	0.0011
			30		3.9	0.05	40.6	0.0128	0.0012
			60		2.9	0.06	113.0	0.0206	0.0005
	Ratio of 1 to 2 at 30 minutes							27.2	1.0
3	2.00	24	15	PCMB	5.7	1.00	590.7	0.1754	0.0016
			30		2.5	0.77	729.2	0.3080	0.0010
			60		0.9	0.58	761.9	0.6440	0.0007
4	2.04	24	15	Saline	7.9	0.04	23.0	0.0050	0.0017
			30		4.6	0.04	36.0	0.0085	0.0011
			60		2.5	0.05	56.3	0.0200	0.0008
	Ratio of 3 to 4 at 30 minutes							35.8	1.0
5	1.82	36	15	PCMB	28.8	1.69	665.4	0.0585	0.0025
			30		13.1	2.20	1109.0	0.1679	0.0019
			60		7.7	2.31	1527.2	0.3000	0.0015
6	1.93	36	15	Saline	20.2	0.06	24.2	0.0029	0.0024
			30		16.6	0.12	60.0	0.0072	0.0020
			60		6.6	0.16	106.6	0.0242	0.0015
	Ratio of 5 to 6 at 30 minutes							23.3	1.0
7	1.8	48	15	PCMB	49.2	0.88	352.0	0.0178	0.0025
			30		35.0	2.41	651.3	0.0688	0.0037
			60		21.3	3.08	1336.5	0.1446	0.0023
8	1.8	48	15	Saline	30.4	0.04	16.0	0.0013	0.0025
			30		26.8	0.16	41.5	0.0059	0.0038
			60		14.3	0.20	87.0	0.0349	0.0023
	Ratio of 7 to 8 at 30 minutes							11.6	1.0

*p-Chloromercuribenzoate.
†Cerebrospinal fluid.

Table II shows the effect of body weight on the concentration of serum and spinal fluid bilirubin and the ratio of SFB/SB. It can be seen that ratio of SFB/SB at 30 minutes, when spinal fluid bilirubin will show the typical response to 48 mg. per kilogram of bilirubin, is progressively decreasing as rabbits' body weight increases. A negative correlation coefficient ($r = -0.91$; d.f. $= 8$; P less than 0.01) was obtained between body weight and ratio SFB/SB at 30 minutes. The ratio SFB/SB of the rabbits weighing about 0.5 and 1.0 Kg. was roughly 2.7 and 1.3 times higher than the rabbits weighing about 2.0 Kg.

TABLE II. EFFECT OF BODY WEIGHT ON THE CONTENT OF SERUM AND SPINAL FLUID BILIRUBIN IN RABBITS AT VARIOUS TIMES AFTER 48 MG./KG. OF BILIRUBIN I.V.

RABBIT NO.	WEIGHT OF RABBIT (KG.)	TIME (MINUTES)	SERUM BILIRUBIN (MG./100 ML.)	CSF BILIRUBIN (MG./100 ML.)	CSF PROTEIN (MG./100 ML.)	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN	RATIO CSF BILIRUBIN TO CSF PROTEIN
9	0.55	15	25.6	0.09	32.5	0.0035	0.0027
		30	18.3	0.19	38.7	0.0103	0.0049
		60	12.4	0.25	54.3	0.0201	0.0046
10	0.55	15	25.1	0.08	30.0	0.0031	0.0027
		30	17.8	0.19	38.7	0.0106	0.0049
		60	12.4	0.26	57.7	0.0209	0.0045
11	0.57	15	28.4	0.08	30.8	0.0028	0.0026
		30	15.8	0.17	34.6	0.0107	0.0049
		60	11.0	0.23	50.7	0.0209	0.0045
12	0.70	15	29.1	0.07	26.9	0.0024	0.0026
		30	15.8	0.15	31.2	0.0071	0.0048
		60	11.0	0.21	43.7	0.0177	0.0048
13	1.00	15	32.1	0.06	24.6	0.0018	0.0025
		30	23.1	0.13	28.8	0.0056	0.0045
		60	10.1	0.19	38.0	0.0186	0.0050
14	1.20	15	33.3	0.06	25.0	0.0018	0.0024
		30	24.0	0.12	26.6	0.0050	0.0045
		60	10.5	0.18	36.0	0.0170	0.0050
15	1.64	15	34.2	0.05	21.7	0.0014	0.0023
		30	24.3	0.11	26.1	0.0045	0.0042
		60	11.3	0.17	30.9	0.0150	0.0055
16	1.80	15	36.3	0.05	21.7	0.0013	0.0023
		30	25.5	0.10	23.0	0.0039	0.0039
		60	11.3	0.12	20.0	0.0096	0.0060
17	1.90	15	33.2	0.05	21.7	0.0012	0.0023
		30	23.2	0.09	24.3	0.0038	0.0037
		60	13.4	0.13	21.6	0.0097	0.0060
18	2.00	15	35.8	0.05	21.2	0.0013	0.0023
		30	25.6	0.10	26.3	0.0040	0.0034
		60	12.7	0.13	21.7	0.0101	0.0060

A correlation was also noted between spinal fluid bilirubin and protein. These two values have been plotted in Fig. 1 from the 10 rabbits in Table II and the 4 in Table I given saline only intracisternally.

Only 15-minute values were used as any bleeding due to the technique would vitiate later protein readings. There was a positive linear correlation ($r = +0.87$; d.f. $= 12$; P less than 0.01).

3. Effect of Sodium Glucuronate.—Five small rabbits weighing about 0.5 Kg. were given 10 per cent sodium glucuronate (The Vitarine Company, New York, N. Y.), 50 mg. per Kg. intravenously, as a single dose just before the intravenous injection of bilirubin (48 mg./Kg.). Five large rabbits weighing about 2 Kg. were given 200 mg./Kg. of it in 3 divided doses, 15 minutes before, just after, and 15 minutes after the intravenous injection of bilirubin (48 mg./Kg.). The results are compared with 5 small and 5 large control rabbits given 5 per cent dextrose in water instead of the glucuronate. The results are shown in Table III. It can be seen that in small rabbits the means of serum bilirubin of the rabbits treated with sodium glucuronate are lower than that of the control rabbits at all times. In contrast the means of spinal fluid bilirubin and the mean ratio of SFB/SB of the rabbits treated with sodium glucuronate are higher than that of the control rabbits. The means of spinal fluid bilirubin of the sodium glucuronate rabbits are roughly 1.5 times higher than that of the control rabbits.

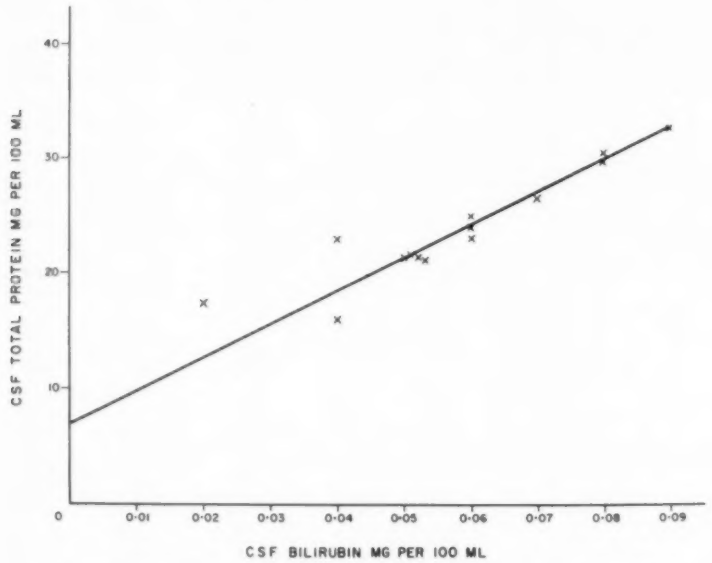

Fig. 1.—A comparison of spinal fluid protein and bilirubin levels at 15 minutes is shown. Data are from the 10 rabbits used in experiment 2 and the 4 used in experiment 1 which were given saline only intracisternally. ($\gamma = +0.87$; d.f. = 12; P less than 0.01.)

In the case of the large rabbits the mean of serum bilirubin at 30 minutes of the rabbits treated with sodium glucuronate is lower than that of the control rabbits but no difference could be noted at 15 and 60 minutes. In addition to the lower serum bilirubin at 30 minutes, the mean of spinal fluid bilirubin and the mean ratio of SFB/SB of the rabbits treated with sodium glucuronate are roughly 1.5 and 2.5 times, respectively, higher than that of the control rabbits at 30 minutes.

Several interesting observations were noted during this series of experiments: (1) If the bilirubin solution was injected rapidly following the injection of 200 mg./Kg. of sodium glucuronate, the rabbits would die suddenly. This could largely be avoided by injecting the bilirubin solution very slowly. (2) The muscle and especially fat tissues among rabbits treated with sodium glucuronate showed yellow staining while none of the control animals showed this effect. (3) In a few rabbits treated with sodium glucuronate from which brains were removed, more yellowish discoloration of brain surface was observed macroscopically on fresh specimen, but no actual yellow staining was left after the tissues were fixed in formalin solution for a week.

TABLE III. EFFECT OF SODIUM GLUCURONATE I.V. ON THE CONTENT OF SERUM AND SPINAL FLUID BILIRUBIN IN RABBITS AT VARIOUS TIMES AFTER 48 ML./KG. OF BILIRUBIN I.V.

GROUP	NO. OF RABBITS STUDIED	TIME (MINUTES)	SERUM BILIRUBIN (MG./100 ML.)‡	CSF BILIRUBIN (MG./100 ML.)‡	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN‡
Small Rabbits* (about 0.5 Kg.)	5	15	16.1 ± 1.7	0.14 ± 0.01	0.0090 ± 0.0012
		30	13.1 ± 1.8	0.32 ± 0.01	0.0318 ± 0.0126
		60	3.5 ± 1.6	0.37 ± 0.01	0.1483 ± 0.0147
Small Rabbits (about 0.5 Kg.) Control	5	15	27.1 ± 1.5	0.08 ± 0.01	0.0029 ± 0.0004
		30	17.3 ± 1.3	0.18 ± 0.02	0.0097 ± 0.0017
		60	11.6 ± 0.6	0.24 ± 0.02	0.0204 ± 0.0016
Large Rabbits† (about 2 Kg.)	5	15	30.6 ± 4.2	0.06 ± 0.03	0.0023 ± 0.0005
		30	18.0 ± 3.7	0.16 ± 0.04	0.0098 ± 0.0002
		60	10.5 ± 1.7	0.21 ± 0.03	0.0203 ± 0.0036
Large Rabbits (about 2 Kg.) Control	5	15	33.9 ± 2.0	0.05 ± 0.00	0.0013 ± 0.0001
		30	24.9 ± 1.2	0.10 ± 0.01	0.0040 ± 0.0002
		60	11.6 ± 1.1	0.14 ± 0.02	0.0060 ± 0.0003

*Sodium glucuronate, 50 mg. per Kg. intravenously just before the bilirubin injection.
†Sodium glucuronate, 200 mg. per Kg. intravenously in 3 divided doses.
‡Expressed as means and standard deviations.

4. Effect of Versenate.—Five rabbits weighing about 2 Kg. were given Versenate in saline (1 ml. = 4 mg.) 44 mg./Kg. intravenously before the intravenous injection of bilirubin (48 mg./Kg.). Five rabbits of about the same size were given the same amount of normal saline, and used as controls.

TABLE IV. EFFECT OF VERSENATE I.V. ON THE CONTENT OF SERUM AND SPINAL FLUID BILIRUBIN IN RABBITS (ABOUT 2 KG.) AT VARIOUS TIMES AFTER 48 MG./KG. OF BILIRUBIN I.V.

GROUP	NO. OF RABBITS STUDIED	TIMES (MINUTES)	SERUM BILIRUBIN (MG./100 ML.)†	CSF BILIRUBIN (MG./100 ML.)†	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN†
Versenate*	5	15	25.4 ± 1.2	0.07 ± 0.00	0.0027 ± 0.0002
		30	15.0 ± 2.3	0.10 ± 0.01	0.0069 ± 0.0010
		60	8.3 ± 2.4	0.20 ± 0.01	0.0255 ± 0.0055
Control	5	15	26.1 ± 1.4	0.07 ± 0.00	0.0027 ± 0.0001
		30	16.3 ± 2.5	0.10 ± 0.01	0.0060 ± 0.0004
		60	8.9 ± 2.8	0.20 ± 0.02	0.0247 ± 0.0050

*Forty-four milligrams per kilogram. (4 mg. in 1 ml. of normal saline.)
†Expressed as means and standard deviations.

From Table IV it can be seen that both the Versenate-treated group and the control group serum bilirubin levels are lower than the control rabbits in

Table III. Probably this is due to the fact that quite a large amount of either Versenate in saline or normal saline (11 ml./Kg.) was given intravenously in addition to the usual intravenous injections needed for the experiment. It would appear that Versenate has no marked effect on all 3 elements: serum bilirubin, spinal fluid bilirubin, and the ratio of spinal fluid bilirubin to serum bilirubin (SFB/SB).

5. Effect of Hykinone.—Hykinone (Abbott's trade name for menadione sodium bisulfite) in doses for 2.5 and 10 mg. was given to each of 5 rabbits intravenously before the bilirubin injection. The results were compared with the control group of Table III in which almost the same amount of 5 per cent dextrose in water had been given as the volume of the Hykinone injection.

Table V shows the effect of intravenous Hykinone (2.5 mg.) on hyperbilirubinemia. The difference between the means of serum bilirubin for the rabbits treated with Hykinone and that of the control rabbits is statistically significant (P less than 0.01) only at 30 minutes. Although spinal fluid bilirubin levels and SFB/SB ratios in the rabbits treated with Hykinone are statistically significantly higher than the control rabbits and show roughly 1.3 and 2 times increase, respectively, the values of spinal fluid bilirubin are at a level where it is known that one cannot place too much reliability on the results. Therefore, it is felt that the results should be interpreted cautiously.

TABLE V. EFFECT OF HYKINONE ON THE CONTENT OF SERUM AND SPINAL FLUID BILIRUBIN IN RABBITS (ABOUT 2 KG.) AT VARIOUS TIMES AFTER 48 MG./KG. OF BILIRUBIN I.V.

GROUP	NO. OF RABBITS STUDIED	TIME (MINUTES)	SERUM BILIRUBIN (MG./100 ML.)‡	CSF BILIRUBIN (MG./100 ML.)‡	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN‡
Hykinone*	5	15	32.7 ± 0.8	0.07 ± 0.00	0.0022 ± 0.0001
		30	22.5 ± 0.9	0.13 ± 0.01	0.0059 ± 0.0007
		60	12.8 ± 0.7	0.19 ± 0.01	0.0152 ± 0.0004
Hykinone†	5	15	41.6 ± 5.2	0.08 ± 0.01	0.0017 ± 0.0002
		30	28.8 ± 3.5	0.11 ± 0.01	0.0038 ± 0.0004
		60	15.0 ± 4.3	0.14 ± 0.02	0.0082 ± 0.0008
Control	5	15	33.9 ± 2.0	0.05 ± 0.00	0.0013 ± 0.0001
		30	24.9 ± 1.2	0.10 ± 0.01	0.0041 ± 0.0002
		60	12.6 ± 1.1	0.14 ± 0.02	0.0060 ± 0.0003

*A single dose of 2.5 mg. was given intravenously.
†A single dose of 10 mg. was given intravenously.
‡Expressed as means and standard deviations.

Table V also shows the effects of intravenous Hykinone (10 mg.) on hyperbilirubinemia. In comparing the two groups the only difference is the mean of serum bilirubin of the rabbits treated with Hykinone at 15 minutes which is 23 per cent (41.6/33.9) higher than that of the control rabbits and the difference is significant (P less than 0.05). In general, the higher doses of Hykinone did not markedly influence the hyperbilirubinemia. However, rabbits injected with 2.5 mg. of Hykinone intravenously survived, whereas all rabbits injected with 10 mg. died. The course of death was not sudden and violent as often seen in rabbits when 200 mg./Kg. of sodium glucuronate was given as a single dose immediately preceding the injection of bilirubin.

6. Effect of Gantrisin.—Five large rabbits weighing about 2 Kg. were given Gantrisin 0.1 Gm./Kg. once daily intramuscularly for 5 consecutive days, and another 5 rabbits of approximately the same size were given the same dose of the drug only on the day before the experiment with intravenous injection of the bilirubin solution, 48 mg./Kg. The results were compared with the 5 control rabbits.

From Table VI it can be seen that the two methods of administering Gantrisin had little actual effect upon the hyperbilirubinemia. However, in comparing the group of rabbits to which Gantrisin was given for 5 consecutive days before the experiment and the control rabbits, it can be seen that the spinal fluid bilirubin concentrations and the ratios SFB/SB are higher in the experimental rabbits than the control. At 30 minutes both factors are 2 times higher in the experimental rabbits than in the controls. This is statistically significant (P less than 0.01).

TABLE VI. EFFECT OF GANTRISIN I.M. ON THE CONTENT OF SERUM AND SPINAL FLUID BILIRUBIN IN RABBITS (ABOUT 2 KG.) AT VARIOUS TIMES AFTER 48 MG./KG. OF BILIRUBIN I.V.

GROUP	NO. OF RABBITS STUDIED	TIME (MINUTES)	SERUM BILIRUBIN (MG./100 ML.)*	CSF BILIRUBIN (MG./100 ML.)*	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN*
Gantrisin†	5	15	35.6 ± 2.0	0.08 ± 0.01	0.0022 ± 0.0005
		30	23.1 ± 1.6	0.20 ± 0.04	0.0088 ± 0.0017
		60	13.8 ± 2.0	0.26 ± 0.05	0.0194 ± 0.0048
Gantrisin‡	6	15	34.1 ± 6.7	0.09 ± 0.04	0.0024 ± 0.0008
		30	24.2 ± 6.1	0.19 ± 0.06	0.0059 ± 0.0024
		60	14.7 ± 5.6	0.27 ± 0.05	0.0201 ± 0.0108
Control	5	15	33.9 ± 2.0	0.05 ± 0.00	0.0013 ± 0.0001
		30	24.9 ± 1.2	0.10 ± 0.01	0.0040 ± 0.0002
		60	11.6 ± 1.1	0.14 ± 0.02	0.0060 ± 0.0003

*Expressed as means and standard deviations.
†Dose of 0.1 Gm./Kg. I.M. was given once daily, 5 days before experiment.
‡Dose of 0.1 Gm./Kg. I.M. was given once daily, one day before experiment.

Ten out of eleven rabbits receiving Gantrisin (listed in Table VI) died. The survivor was one of those who only received a single injection of Gantrisin. This rabbit showed lower serum bilirubin levels of 24.9, 14.9, and 6.8 mg. per 100 ml. at 15, 30, and 60 minutes, respectively. The spinal fluid bilirubin levels at the corresponding times were 0.07, 0.25, and 0.29 mg. per 100 ml. Brains of several dead rabbits were removed and macroscopically showed the same findings as in rabbits treated with sodium glucuronate.

7. Effect of Anoxemia.—Five small rabbits weighing about 0.5 Kg. were kept in an incubator 30 minutes before the intravenous injection of 24 mg./Kg. of bilirubin. A constant flow of 10 per cent oxygen and 90 per cent nitrogen mixture was run through the incubator. As controls, another 5 rabbits of approximately the same size in an ordinary environment were given the same dose of bilirubin. Table VII shows the results. The difference between the serum bilirubin means for the experimental rabbits and that of the control group is significant (P less than 0.05) only at 15 minutes. The ratio of SFB/SB at 15 minutes is 1.6 times higher in the experimental rabbits than the control group (P less than 0.01).

TABLE VII. EFFECT OF ANOXEMIA ON THE CONTENT OF SERUM AND SPINAL FLUID IN RABBITS (ABOUT 0.5 KG.) AT VARIOUS TIMES AFTER 24 MG./KG. OF BILIRUBIN I.V.

GROUP	NO. OF RABBITS STUDIED	TIME (MINUTES)	SERUM BILIRUBIN (MG./100 ML.)*	CSF BILIRUBIN (MG./100 ML.)*	RATIO CSF BILIRUBIN TO SERUM BILIRUBIN*
Experimental animals†	5	15	8.8 ± 1.2	0.10 ± 0.01	0.0112 ± 0.0010
		30	3.8 ± 1.0	0.20 ± 0.02	0.0613 ± 0.0236
		60	1.7 ± 0.8	0.31 ± 0.04	0.2267 ± 0.1271
Control	5	15	10.8 ± 1.2	0.07 ± 0.01	0.0069 ± 0.0012
		30	3.8 ± 0.5	0.20 ± 0.01	0.0541 ± 0.0058
		60	2.0 ± 0.6	0.33 ± 0.05	0.1783 ± 0.0404

*Expressed as means and standard deviations.

†Kept for 30 minutes in an incubator with a constant flow of 10 per cent O_2 and 90 per cent N_2 mixture just prior to the experiment.

DISCUSSION

Recently, Nasralla and associates,[10] have shown that there is a severalfold increase in the permeability of the blood-spinal fluid barrier for bilirubin in the newborn period. Furthermore, this difference was most marked among the premature infants weighing 4½ pounds or less. Since these findings may be important in elucidating the pathogenesis of kernicterus, it appeared important to undertake more extensive studies using bilirubin loads in rabbits.

The first series of studies were centered around showing the maximal changes when the blood-spinal fluid barrier was damaged by a toxic agent. Ernster and his co-workers[9] have found that the blood-spinal fluid barrier for bilirubin can be markedly reduced by the intracisternal injection of p-chloromercuribenzoate (PCMB) in a rabbit. They postulated that this compound had an inhibitory effect upon sulfhydryl-dependent enzymes since its action can be reversed by glutathione.[13] The present studies confirmed their findings.

We then attempted to show that there was a greater permeability of the blood-spinal fluid barrier among younger animals than mature ones using a bilirubin load. We felt that it was important to show the same phenomena in animals who were not jaundiced initially because the physiologic jaundice in newborn infants might influence the blood-spinal fluid barrier due to adaptation. Assuming that the ratio SFB/SB is a measure of the permeability of the blood-spinal fluid barrier, then in the limited number of rabbits studied we demonstrated that the barrier was 2.7 times as permeable in rabbits weighing 0.5 Kg. as those of 2.0 Kg. As the body weight of the rabbits increased, the blood-spinal fluid barrier became increasingly effective.

Next, we felt it was important to test the effects of various agents which have been known to have had an effect upon hyperbilirubinemia or the incidence of kernicterus in newborn infants. In 1958, Danoff and associates[14] reported that the administration of sodium glucuronate, both orally and intravenously, to newborn infants with hyperbilirubinemia resulted in a fall of indirect reacting bilirubin. However, 14 cases treated in Boston by Jeliu and associates[15] failed to confirm their results. Johnson and co-workers[16] have studied the effects of sodium glucuronate upon the Gunn rat, a strain of animals who remain jaundiced throughout life because of a genetically determined deficiency of glucuronyl transferase. She was unable to demonstrate any differences in 20 adult rats, but in 5 young animals the bilirubin level

appears to have been lowered. In the latter group, kernicterus still occurred and, in fact, appeared to be somewhat more intense. In our own laboratory we have demonstrated through the use, in a dog, of C^{14}-labeled sodium glucuronate that it does not take part in the conjugation of bilirubin, because no radioactivity could be detected in the bilirubin obtained from the bile duct of the dog.[17] We have also found that there is an increase of bilirubin in muscle following the administration of sodium glucuronate in jaundiced rabbits.[18] The present data would suggest that the cerebrospinal fluid would be another means of disposal of the bilirubin. The high mortality rate and increased yellowish discoloration of the brain surface following the injection of sodium glucuronate would suggest that this was not a physiologic means for reducing hyperbilirubinemia.

Some workers have suggested that sodium glucuronate may act as a chelating agent and it appears important to test the action of Versenate upon this system. The present data showed that it had no marked effect upon the serum and spinal fluid bilirubin levels. The failure to show any effect is not unexpected since Childs[19] has previously shown that bilirubin glucuronide exists in the serum as a bilirubin-metal complex, and the addition of Versenate to serum will bring about the conversion of most of the direct pigment to the indirect form. Since the injected bilirubin is already in the indirect form, no further change would be expected to occur.

To date only a vitamin K analogue sodium menadione diphosphate (Synkavit) has been shown to be capable of causing hyperbilirubinemia when administered to newborn infants. Recently Lucey[20] observed hyperbilirubinemia of newborn infants associated with the administration of a large intravenous dose of Hykinone to mothers before delivery. Two studies[21, 22] have demonstrated that Hykinone is more toxic than Synkavit in animals. Hykinone also contains 1.3 times as much menadione per milligram of dose as Synkavit. Menadione is thought to be the probable toxic portion. Several explanations have been proposed to explain this toxicity, these include increased formation of Heinz bodies,[23, 24] reducing glutathione content of cells,[25] possible competition of a glucuronyl transferase[26] and hepatocellular damage.[27] In the present study, we found that Hykinone in small doses of 2.5 mg. did not cause any deaths even though the ratios of SFB/SB increased. On the other hand, using a larger dose (10 mg.), all of the treated rabbits died despite the fact that the only difference between the treated group and the control group was a slight increase in the concentration of serum bilirubin levels at 15 minutes in the treated group. Asteriadou-Samartzis and Leikin[28] have reported that 25 mg. doses of vitamin K_1 when given to premature or full-term infants intravenously did not have a hyperbilirubinemic effect. They speculated that it may be that the intravenous vitamin K_1 preparation given in a single dose was quickly converted to prothrombin, eliminating any lasting hyperbilirubinemic effect. We are still unclear about the action of Hykinone on bilirubinemia.

Silverman and associates[29] have shown that there is an increased incidence of kernicterus among premature infants given penicillin and Gantrisin as compared with those given oxytetracycline prophylactically. Subsequently, Harris

and associates[30] noted that the treatment of premature infants with Gantrisin and sulfadiazine lowered the average serum bilirubin level by 30 per cent. Although we were unable to show any significant lowering of serum bilirubin levels in the rabbits treated with Gantrisin which died, only one rabbit receiving the Gantrisin regimen for one day survived and showed lower serum bilirubin levels than controls. Furthermore, there was an increased permeability of the blood-spinal fluid barrier for bilirubin among the animals treated with Gantrisin. This would seem to confirm the hypothesis proposed by Lucey[31] that Gantrisin has a toxic effect upon the transport mechanism for bilirubin across the blood-brain barrier.

We finally turn to the problem of the effect of anoxemia upon bilirubinemia. In a recent paper, Miller and Reed[32] showed a correlation between the degree of hyperbilirubinemia and increased respiratory rate among premature infants. Gröntoft,[33] in a study which included the anatomic evaluation of 319 perinatal deaths, concluded that anoxic damage contributed significantly to the postmortem evidence of increased permeability of blood-brain barrier in the immature infant. Because of technical difficulties, the degree of anoxia in the present studies was not as marked as one would desire. Nevertheless, there is some suggestive evidence that there is an increased permeability of the blood-spinal fluid barrier at 15 minutes in the experimental rabbits as compared with the controls.

From these studies, it would appear that the permeability of the blood-spinal fluid barrier is influenced by the weight of the animal and perhaps to a lesser extent by the degree of anoxemia. It would appear that the lowering of serum bilirubin levels following sodium glucuronate and Gantrisin is due in part to an increased flow of bilirubin to the spinal fluid. The influence of Synkavit is somewhat more difficult to assess, but it, too, may play a role.

Two theories exist as to how the staining of the brain occurs in kernicterus. Some workers believe that the increase of bilirubin levels in the blood directly causes the pigment to be deposited in the brain. Vogel[34] has suggested that kernicterus occurs because there is an increased permeability of the blood-brain barrier during early infancy. This bilirubin is then reduced within the brain to mesobilirubin which gives rise to the cerebral pigmentation in kernicterus. The data from the present study would lend further strength to the concept that the level of bilirubin in the spinal fluid must in part play a role in the pathogenesis of kernicterus.

SUMMARY

In order to investigate blood-spinal fluid barrier, simultaneous samples of blood and spinal fluid were obtained from 79 rabbits of varying weights (0.41 to 2.2 kilogram) at 15, 30, and 60 minutes after intravenous injection of varying doses (12 to 48 mg./Kg.) of the bilirubin solution in combination with pretreatment with p-chloromercuribenzoate (PCMB), sodium glucuronate, Versenate, Hykinone, and 10 per cent oxygen and 90 per cent nitrogen mixtures. The permeability of the blood-spinal fluid barrier was expressed as the ratio of spinal fluid bilirubin to serum bilirubin (SFB/SB).

We have confirmed the findings of Ernster and his co-workers[9] that the blood-spinal fluid barrier for bilirubin can be markedly reduced by the intracisternal injection of PCMB in rabbits.

A negative correlation coefficient ($r = -0.9$) was obtained between body weight and SFB/SB ratios 30 minutes after the rabbits were given 48 mg./Kg. of bilirubin.

In small rabbits sodium glucuronate (50 mg./Kg. in a single dose) induced lower serum bilirubin levels and a 1.5 fold increase of spinal fluid bilirubin. All rabbits receiving a large dose of Hykinone (10 mg. intravenously) and 10 out of the 11 rabbits receiving the Gantrisin treatment died after the intravenous injection of 48 mg./Kg. of bilirubin. Versenate and Hykinone (2.5 mg. intravenously) showed no marked influences on bilirubinemia.

Anoxemia appeared to influence the spinal fluid bilirubin–serum bilirubin ratio at 15 minutes only. The significance of these findings in the pathogenesis of kernicterus was discussed.

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AN APPRAISAL OF THE LATEX TEST FOR RHEUMATOID ARTHRITIS

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IN RECENT years interest has been aroused in the ability of the serum from patients with rheumatoid arthritis to agglutinate a variety of particles or enhance their agglutination by other agents. Several tests have been developed which depend on this phenomenon and have usually employed partially sensitized sheep red cells as the indicator of agglutination-enhancing activity. For a routine laboratory, however, sheep cells are not an ideal reagent. They must be kept sterile, used within a few days of withdrawal, and each batch must be standardized for capacity to be agglutinated. The use of polystyrene latex particles coated with human gamma globulin as the indicator of agglutination[1, 2] has the advantages of simplicity and stable reagents.

In both the sheep cell and the latex tests, the serum of 1 in 3 or 4 patients with clinically typical rheumatoid arthritis fails to show agglutination. If, instead of whole serum, an euglobulin fraction is used, agglutination of sensitized sheep cells occurs in about 9 out of 10 cases of rheumatoid arthritis and only 2 per cent of the controls.[3] A further modification, giving a still more sensitive but less specific test, consists of examining the euglobulin fraction for the presence of a factor which inhibits the agglutinating activity of known rheumatoid serum.[3] This inhibitory factor is said to be absent from rheumatoid serum almost invariably and to be present in most normal sera.

It seemed that a combination of these tests might be found which was reasonably simple and yet possessed high accuracy. We have, therefore, used the latex technique to estimate the content of agglutinating and inhibitory factors in the euglobulin fraction and the whole serum of some healthy subjects, some patients with rheumatoid arthritis and some with other diseases. Since the value of such tests can only be established by the pooling of many observations, our results are briefly reported.

METHODS

Patients.—The patients with rheumatoid arthritis, other forms of arthritis, or related diseases were outpatients of this clinic. The diagnosis was made by other members of the staff without reference to the latex test. The rheumatoid arthritis patients all fell within

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the "definite" or "probable" categories of Ropes and associates.[4] Normal sera were obtained from students and staff of the medical school and control sera came from outpatients who attended the hematology or cancer detection clinics and were free from arthritic manifestations on routine clinical examination.

Procedure.—Sera were stored at 4° C. and were not inactivated before testing; many were re-examined after inactivation at 56° C. for 30 minutes. The tests were performed by the technique of Singer and Plotz,[1] using their glycine buffer.[5] All solutions were refrigerated and, apart from the latex suspension, were filtered before use since fragments of fibrin or dust could simulate slight agglutination. Doubling dilutions of serum or euglobulin from 1:20 to 1:320 were examined, and the final volume was always 3 ml. in the reaction tubes (2 ml. of serum dilution and 1 ml. of latex-gamma globulin reagent). Positive and negative controls were always included.

Euglobulin was separated by dialysis of 2 to 3 ml. serum against a citrate-phosphate buffer at pH 5.8 in the cold for 48 hours.[3] The resulting precipitate was washed once with buffer and dissolved in a volume, equal to the original serum specimen, of 0.9 per cent saline containing 0.3 M glycine and brought to pH 7.3 by addition of dilute sodium hydroxide. A clearer solution of the precipitated euglobulin was obtained in this way than when physiologic saline or phosphate buffer was used and this facilitated recognition of slight agglutination.

Inhibitor was sought by a simple adaptation of Ziff's technique[3] for sheep cells. In 3 tubes were placed 1 ml. volumes of 1:7, 1:14, and 1:28 dilutions of the euglobulin under test. To each tube was added 1 ml. of known positive rheumatoid serum in a dilution 8 times stronger than the highest dilution which caused agglutination. Thus, dilutions of 1:14, 1:28, and 1:56 of the euglobulin were created before addition of 1 ml. of latex-gamma globulin reagent to each tube. The known positive sera were titrated frequently; their titer usually remained constant for several weeks at 4° C.

Inhibition of agglutination by a dilution of 1:14 or more was taken as normal.[3] Although the decision concerning normality rests on the reaction at this dilution, the estimation of inhibitor in 3 dilutions is probably wise since the test can be difficult to read, and comparison of the 3 tubes may give helpful internal evidence. Normal euglobulin will show either increased agglutination as the dilution increases (i.e., as inhibitor is diluted) or equal agglutination in all tubes if inhibitor is absent. Rheumatoid euglobulin typically shows the strongest agglutination in the first tube, where the largest amount of agglutinating activity from the euglobulin is added to the known positive serum.

Three grades of agglutination were recognized: + = fine, only visible with certainty by hand lens; ++ = moderate, clearly visible to the naked eye; +++ = coarse, obvious clumps with clear background. In doubtful cases rereading the tubes after overnight refrigeration was often helpful. In tests involving euglobulin, tapping the centrifuged precipitate may produce large strands which readily break up with further tapping, forming a milky background containing a few clumps. This does not denote agglutination. The appearance of the background is more important than the presence of a few aggregates and may be likened to astronomical pictures. Thus, Plates 25 (background), 31, and 51 of Hoyle's book[6] are excellent illustrations of the hand lens appearance of negative, weak positive, and strong positive agglutination tests.

RESULTS

Table I shows the number of patients in each disease group whose serum and euglobulin showed agglutination at 1:20 and 1:160 dilutions. The last column gives the number whose euglobulin fraction (not inactivated) contained inhibitory activity at 1:14 or higher dilution.

Rheumatoid Arthritis.—These patients showed a much higher proportion of positive tests than any other group and exhibited a sex difference. All the men showed agglutination at a dilution of 1:20 both of serum and of euglobulin;

only one serum failed to show agglutination at 1:160. Out of 57 women, agglutination at 1:20 occurred with 45 sera (79 per cent) and 53 euglobulin specimens (93 per cent); 36 sera and 44 euglobulin fractions reached a titer of 1:160. The differences in the proportions of men and women who showed agglutination were not likely to be fortuitous either at 1:20 or 1:160 dilutions of serum or at 1:160 dilution of euglobulin.

Although low titer and negative tests were more frequent in patients whose disease was clinically quiescent, this could readily have been a chance association, and there was no significant correlation between any clinical feature and positive or negative tests. Four women (Table II) failed to show any agglutination in serum or euglobulin. Two had largely "burnt out" arthritis with residual deformity and two had typical moderately active disease. One of the latter was strongly suspected of having systemic lupus erythematosus during the stormy onset of her disease but this remained unproved and the subsequent course has been that of rheumatoid arthritis.

Two women with rheumatoid arthritis and widespread psoriasis failed to show any agglutination and both had inhibitor which was destroyed by inactivation.

TABLE I. AGGLUTINATION RESULTS IN VARIOUS DISEASES

DISEASE	NO.	SERUM		EUGLOBULIN		INHIBITOR PRESENT
		1:20	1:160	1:20	1:160	
Rheumatoid arthritis						
Men	19	19	18	19	19	0
Women	57	45	36	53	44	9
p difference		$0.05>p>0.02$	0.01	—	$0.05>p>0.02$	
Juvenile	8	2	0	3	1	5
With psoriasis	2	0	0	0	0	2
Osteoarthritis	27	8	0	2	0	23
Other arthritides	30	5	0	1	0	26
Systemic lupus erythematosus	19	6	3	5	2	11
Collagen diseases	6	3	0	5	2	1
Minor rheumatism	14	1	0	0	0	10
Healthy	48	4	0	3	0	41
Other diseases	70	7	0	2	0	65

The age and duration of disease were not important. Nine patients (4 men and 5 women) were tested between 3 and 12 months after the onset of their disease. The men and two of the women gave titers of 1:160 in serum and euglobulin and 2 other women gave lower titers. Eighteen women had arthritis of at least 10 years' duration and 10 of these showed agglutination at 1:160 in serum or euglobulin, 6 gave lower titers and 2 were negative.

No correlation with sedimentation rate was apparent. Five women had sedimentation rates of 20 mm. or less in 1 hour. One gave no serum agglutination and two only low titers. Four out of five euglobulin titers reached 1:160, the other being negative. The 4 women who failed to show any agglutination had elevated sedimentation rates (Table II).

Treatment did not apparently affect agglutination results. At the time of the test 7 patients were taking prednisone, 11 antimalarial drugs, and 4 were

having weekly gold injections; their results did not differ materially from those of patients taking only salicylates. Complete absence of agglutination was not associated with the taking of any particular drug (Table II).

TABLE II. PATIENTS GIVING NO AGGLUTINATION

AGE	SEX	DURATION	RACE	S. R.*	INHIBITOR†		DAILY THERAPY	CLINICAL ACTIVITY OF DISEASE
					EUGLOBULIN	INACTIVE EUGLOBULIN		
45	F	5	C	65	<14		Prednisone 7.5 mg.	Moderate
40	F	1	C	61	<14		Prednisone 7.5 mg. Chloroquine 750 mg.	Moderate
61	F	8	W	36	56	<14	Aspirin	Quiescent
63	F	37	W	77	56	<14	Aspirin	Quiescent

*S. R. = Sedimentation rate (Westergren), millimeters per 1 hour.
†Figures are the reciprocals of the highest titer.

Inhibitory factor was absent from the euglobulin fraction of all the men. It was present at 1:14 or higher dilution in 9 women but was regularly destroyed by inactivation.

Juvenile Rheumatoid Arthritis.—This group contained 7 children, and one man with inactive disease 34 years after the onset at the age of 9. The adult and 3 children showed no agglutination; 2 other children gave low titers in euglobulin only, and 1 reached a titer of 1:160. Inhibitor was present in 5 patients; in the adult and in one child it persisted after inactivation.

Osteoarthritis.—Two men and 6 women exhibited agglutination in serum, only 4 reaching 1:40 dilution. Two women, both exhibiting negative sera, gave agglutination with euglobulin; one (1:20) also had Paget's disease of bone and the other (1:40) had a gastric ulcer. Inhibitor was present in the euglobulin of all the patients with low titer serum agglutinins. It was absent after inactivation in one of the 4 samples tested.

Other Arthritides.—This group is broken down in Table III. One patient each with rheumatic fever, gonococcal arthritis, atypical spondylitis, and traumatic synovitis gave low titer agglutination in serum only. One patient with gout had titers of 1:40 in serum and euglobulin with absent inhibitor; 3 months later no agglutination occurred but inhibitor remained absent.

TABLE III. OTHER ARTHRITIDES

DISEASE	NUMBER OF PATIENTS	NUMBER SHOWING AGGLUTINATION		INHIBITOR PRESENT AT 1:14
		SERUM	EUGLOBULIN	
Ankylosing spondylitis	4	0	0	4
Rheumatic fever	7	1 (80)	0	7
Gout	7	1 (40)	1 (40)	4
Infective arthritis	5	1 (20)	0	5
Traumatic arthritis	2	1 (20)	0	1
Miscellaneous	5	1 (20)	0	5

Figures in parentheses are the reciprocals of the titers attained.

Systemic Lupus Erythematosus.—Three patients gave high and three gave low titers in serum. One high and two low titer sera were accompanied by a

negative euglobulin test. Inhibitor was present in 11 patients but was not sought routinely after inactivation. There was poor correlation between the degree of joint involvement and the result of the test. Fourteen patients had arthralgia or mild or transient arthritis as part of their disease, and 5 of these gave some agglutination in serum or euglobulin. Five patients had a chronic arthritis of rheumatoid type; 3 of these showed agglutination, 2 in high titer.

Collagen Diseases.—Two patients with dermatomyositis both gave low titer agglutination in serum and euglobulin with absence of inhibitor. There were 4 patients with scleroderma of whom 3 gave agglutination in euglobulin and one in serum also; 3 lacked inhibitor.

Minor Rheumatic Diseases.—This group contained 3 patients with calcific tendinitis, 4 with minor myalgia, 4 with psychogenic rheumatism, and 3 with miscellaneous disorders. One of the myalgic patients had low titer agglutination in serum only.

Healthy Subjects.—Agglutination in serum only was shown by 4 subjects and in euglobulin by 3 others; in one the titer reached 1:80. Inhibitor was absent from the euglobulin fraction of 7 subjects, including the 3 showing agglutination.

Miscellaneous Diseases.—This group yielded 7 positive sera (10 per cent) not accompanied by euglobulin agglutination. Four sera only gave weak agglutination to 1:20; the other patients' diagnoses were cystocele (1:40), thalassemia major (1:80), and anxiety state (1:80). Inhibitor was present in 65 out of 70 patients, including all those who exhibited any agglutination.

Comparison of Titer and Degree of Agglutination in Controls and Rheumatoid Patients.—Table IV compares the incidence of various agglutination titers in adult rheumatoid arthritis patients with the incidence in unrelated conditions (i.e., osteoarthritis, other arthritides, minor rheumatism, other diseases, and healthy subjects). The lowest titer giving no false positives is 1:160 both in serum and euglobulin; this titer in euglobulin shows all the men and 77 per cent of the women with rheumatoid arthritis as positive. The proportion of positive tests in women with rheumatoid arthritis increases at lower titers, but none gives 100 per cent of positive tests and the incidence of false positives rises materially.

TABLE IV. AGGLUTINATION TITERS IN CONTROL AND RHEUMATOID SUBJECTS

TITER REACHED	RHEUMATOID ARTHRITIS (19 M, 57 F)				CONTROL SUBJECTS (86 M, 103 F)			
	SERUM		EUGLOBULIN		SERUM		EUGLOBULIN	
	M	F	M	F	M	F	M	F
<1:20	0	12	0	4	74	90	80	101
1:20	0	5	0	2	7	8	2	1
1:40	0	2	0	2	2	4	3	0
1:80	1	2	0	5	3	1	1	1
1:160	18	36	19	44	0	0	0	0

Table V shows the number of patients who exhibited the 3 degrees of agglutination at 1:20 dilution. Apart from two patients with systemic lupus erythematosus, marked agglutination (+++) occurred only with the sera and euglobulin fractions of patients with rheumatoid arthritis. A few patients in all

other disease groups showed moderate agglutination (++) in serum, but in euglobulin the only ones to give moderate agglutination were a patient with gout, whose test was completely negative on repetition 3 months later, one with osteoarthritis and a gastric ulcer, and 3 patients with scleroderma. In the inhibition procedure, marked agglutination at 1:14 dilution was seen in 12 of 19 men and 28 of 57 women with rheumatoid arthritis, in one boy with Still's disease, and in one woman with systemic lupus erythematosus. It did not occur in any other disease group.

TABLE V. DEGREE OF AGGLUTINATION

DISEASE	NO.	DEGREE OF AGGLUTINATION AT 1:20					
		SERUM			EUGLOBULIN		
		+++	++	+	+++	++	+
Rheumatoid arthritis							
Male	19	13	5	1	16	3	0
Female	57	15	21	9	30	18	5
Juvenile	8	0	1	1	1	0	2
With psoriasis	2	0	0	0	0	0	0
Osteoarthritis	27	0	3	5	0	1	1
Other arthritides	30	0	2	3	0	1	0
Systemic lupus erythematosus	19	2	2	2	1	0	4
Collagen diseases	6	0	1	2	0	3	2
Minor rheumatism	14	0	1	0	0	0	0
Healthy	48	0	1	3	0	0	3
Other diseases	70	0	5	2	0	0	2

Effects of Inactivation.—Table VI shows the results of inactivating a number of euglobulin fractions at 56° C. for 30 minutes. All the rheumatoid specimens lacked inhibitory activity at 1:14 dilution after inactivation. Of the specimens picked at random from among the other disease groups, more than one-third lost inhibitor activity during inactivation.

TABLE VI. EFFECT OF INACTIVATION UPON INHIBITOR

SPECIMEN	TOTAL	NUMBER OF EUGLOBULIN SPECIMENS CONTAINING INHIBITOR AT VARIOUS DILUTIONS						
		BEFORE INACTIVATION			AFTER INACTIVATION			
		1:14	1:28	1:56	<1:14	1:14	1:28	1:56
Rheumatoid arthritis	9	1	4	4	9	0	0	0
Systemic lupus erythematosus	4	1	1	2	2	0	0	2
Other arthritides	10	4	2	4	2	2	2	4
Controls	45	13	5	27	17	9	9	10

TABLE VII. EFFECT OF INACTIVATION ON AGGLUTINATION TITER

SPECIMEN	TOTAL	BEFORE INACTIVATION			AFTER INACTIVATION		
		<1:20	1:20-1:80	>1:80	<1:20	1:20-1:80	>1:80
Serum							
Rheumatoid arthritis	9	7	2	0	5	4	0
Controls	9	8	1	0	7	2	0
Euglobulin							
Rheumatoid arthritis	5	4	1	0	3	2	0
Controls	20	19	1	0	17	3	1

It seemed possible that, after destruction of inhibitor by inactivation, some sera or euglobulin fractions, previously negative, might show agglutination. Table VII shows the effect of inactivation upon some low titer or negative sera

and euglobulin fractions. All the euglobulins had inhibitor present before and absent after inactivation but inhibitor was not estimated in all the sera. After inactivation low titer agglutination appeared in the serum of one control and two rheumatoid patients and in the euglobulin of two controls and one rheumatoid patient. Only one patient, a girl with sarcoidosis histologically demonstrated in lymph glands and kidney, achieved a titer of 1:320 in euglobulin after inactivation; she had no clinical or x-ray evidence of bone or joint involvement.

DISCUSSION

The present results confirm previous observations[2, 7, 8-10] that the latex test is comparable with the more refined forms of the sheep cell test. Positive tests were given by the serum of 83 per cent of our patients with rheumatoid arthritis, a higher proportion than other workers have reported. Thus, Plotz and Singer[2] found the serum of 71 per cent of patients to be positive; Thomas and associates[7] found 72 per cent and Bartfeld and associates[8] found 68 per cent of sera to be positive. Our higher proportion of positive tests might be due to more rigid selection of cases but, since Bartfeld[8] obtained only 68 per cent of positive serum tests from definite[4] rheumatoid patients, this explanation appears untenable. Another possibility is that our series contains an excess of men, who gave a higher incidence of positive tests than women. This cannot be excluded as the sex ratios of the other series are not stated.

Ziff and colleagues[3] found that the sensitivity of the sheep cell test was increased by employing an euglobulin fraction of serum, positive results occurring in 92 per cent of rheumatoid patients. Our figures confirm that increased sensitivity is also obtained with euglobulin in the latex test[9]; use of a globulin fraction, separated by precipitation with 1.64 M ammonium sulfate, may also increase sensitivity.[5]

A stricter criterion of positivity has been advocated, namely, agglutination at a dilution of 1:160.[8] This reduces the sensitivity of the test particularly in women, and euglobulin remains superior to serum. Specificity is considerably enhanced, however; only 2 of our nonrheumatic patients exhibited this titer, both in serum, and repetition showed that one was a technical error. The other occurred in a youth with thalassemia major, and may represent a false positive test produced by jaundice.[18] However, 3 repetitions yielded no higher titer than 1:80 and the euglobulin fraction regularly lacked agglutinating activity. Like Ziff and associates,[3] we have not encountered a positive serum associated with a negative euglobulin test in patients with rheumatoid arthritis.

The strength of agglutination was of some diagnostic help, in that gross agglutination was only seen in rheumatoid arthritis or systemic lupus erythematosus; however, it was by no means constant. A moderate degree of agglutination in the first euglobulin tube was fairly specific, only occurring twice outside the groups of patients with rheumatoid arthritis or collagen diseases.

A lower incidence of positive tests in women has previously been observed with the sheep cell[12] and latex tests[11] and is unexplained. Among any group of patients thought to have rheumatoid arthritis, a few, mainly women, will

eventually prove to have systemic lupus erythematosus. Since we found positive tests much less frequently in established cases of this disease, even when joint involvement was obvious, it may be that some of our "rheumatoid" women with negative tests really have systemic lupus erythematosus (although all have had negative examinations for L.E. cells).

Most investigators have not found any significant correlation between agglutination tests and disease activity or sedimentation rate, although Ball[12] found significantly fewer positive sheep cell tests in inactive cases. We have seen only one patient's tests change from strongly positive to negative in serum and euglobulin; this happened during a complete remission of more than a year's duration. The agglutination tests seem to become positive early in the disease[3] and only revert to negative if a very marked remission occurs. As with the sheep cell test,[12, 13] therapy seems to exert little effect on the latex test.

Ziff and colleagues[3] found that the inactivated euglobulin fraction of 96 per cent of control subjects showed inhibitory activity against agglutination by positive rheumatoid serum, whereas euglobulin from rheumatoid patients never exhibited such activity. With serum, the difference was less definite,[14] as we also have observed. Hall and colleagues[9] found inhibitor in only one of 177 rheumatoid euglobulin fractions. A few of our patients possessed inhibitor before inactivation of the euglobulin but it was uniformly absent afterward. However, our controls also frequently lacked inhibitor so that, although the presence of inhibitor at 1:14 dilution is a strong point against the diagnosis of rheumatoid arthritis, its absence by this technique lends no material weight to the diagnosis.

It has been noted[15] that some negative rheumatoid sera become positive after inactivation. Six of our women who gave negative agglutination tests all had inhibitor in their serum. This was destroyed by inactivation but the titer of agglutination was not thereby enhanced. Similarly, destruction of the inhibitor which is present in some euglobulin fractions made little difference to agglutination results, the incidence of positive tests rising slightly both in rheumatoid patients and controls. Only once did a negative test become strongly positive after inactivation; this patient had sarcoidosis, an occasional cause of false positive tests.[16]

The occurrence of some agglutination in one-third of the patients with systemic lupus erythematosus and in most of those with other "collagen diseases" makes the test of little help in differentiating rheumatoid arthritis from this group. However, 3 of the group showed agglutination in serum only, a phenomenon not seen in rheumatoid arthritis, and the only two high titers in euglobulin occurred in patients who had typical rheumatoid arthritis for several years before other systems were involved and numerous L.E. cells became demonstrable. Positive tests in systemic lupus erythematosus seem to be less common with latex[5, 9] than with sheep cells, for which an average figure of 48 per cent of positives is given.[17]

Low titer agglutination in serum only was common in our osteoarthritic patients. It seems unlikely that they had mild rheumatoid arthritis, predisposing to degenerative change, for none of their hand x-rays showed evidence

of rheumatoid arthritis and, as noted earlier, agglutination in serum only is not seen in this disease. Furthermore, Lawrence and Ball[19] found no increased prevalence of osteoarthritis in the relatives of rheumatoid patients, although rheumatoid arthritis was 4 times more common in these relatives than in control subjects. Thus, mild rheumatoid arthritis is probably not an important predisposing case of osteoarthritis.

The occurrence of agglutination in 6 per cent of control sera and 30 per cent of osteoarthritic sera at 1:20 dilution clearly indicates that a higher titer must be chosen as "positive," and the value of 1:160[8] is acceptable. This level of agglutination would give no false positives among our patients, except for the group with collagen diseases, but it would exclude a material number of women with rheumatoid arthritis. Using euglobulin, agglutination at 1:40 might at least be considered very suggestive, for only 2½ per cent of the patients outside the groups with rheumatoid arthritis and collagen diseases attained this level; at this titer, 89 per cent of the female rheumatoid patients would rank as positive, compared with 77 per cent at 1:160. Clearly the level chosen as positive remains to some extent a matter of personal choice and depends whether sensitivity or specificity is considered more important.

We conclude that the latex test is the equal of the sheep cell test in the diagnosis of rheumatoid arthritis and that its technical advantages and substitution of a biologically inert particle for the sheep red cell make it preferable. Its frequent diagnostic failure in juvenile rheumatoid arthritis and the overlap in patients with collagen diseases remain as challenges to investigation and improvement. The test offers an additional parameter for comparing groups of patients from different centers and is useful in isolating unusual or atypical types of chronic inflammatory arthritis, thereby encouraging their study and avoiding indiscriminate lumping under an overstretched diagnostic label of rheumatoid arthritis.

SUMMARY

The latex test has been applied to the serum and to an euglobulin fraction of the serum of some healthy subjects, some patients with various rheumatic disorders, and some with nonrheumatic diseases. The test appears to be at least as accurate in the diagnosis of rheumatoid arthritis as the sheep cell techniques and it has some technical advantages.

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THE EFFECT OF INTRINSIC FACTOR ON THE HEPATIC UPTAKE OF VITAMIN B_{12} FOLLOWING INTRAVENOUS INJECTION

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MILLER and associates,[1, 2] Latner and Raine,[3] and Herbert[4, 5] observed that intrinsic factor (IF) can enhance, in vitro, the uptake of radioactive vitamin B_{12} by liver slices and homogenates. Miller,[2] who first described this phenomenon, postulated that the IF acts upon tissue receptor proteins and thereby accelerates the uptake of vitamin B_{12}. No study, in vivo, on the participation of IF in the deposition of absorbed vitamin B_{12} has been reported. In this paper, data are presented to demonstrate the effect of intrinsic factor preparations on the vitamin B_{12} uptake by liver and some other organs following an intravenous injection of a B_{12} and intrinsic factor complex.

MATERIALS AND METHODS

A. Rats Used.—Adult rats of either the McCollum strain from our own colony or Sprague-Dawley strain from various other laboratories were used. The latter group of rats were acclimated for at least one week on our stock diet before use.

B. Materials Used.—Two preparations of intrinsic factor of different potencies were supplied to us through the courtesy of the Lederle Laboratories Division, American Cyanamid Company. Preparation A (WES 727) had an IF activity of one U.S.P. unit per 1 to 2 mg., if admixed with 15 μg B_{12}, and was completely soluble in a normal saline solution. Preparation B (WES 519) was active at 100 mg. per U.S.P. unit.* This preparation was not entirely soluble in a normal saline solution and an active extract was made by adjusting a saline suspension to pH 8.5. The mixture was centrifuged and the clear supernatant fluid was used for study. Unless otherwise stated, 40 mμg of Co^{60}-labeled vitamin B_{12} with a specific activity of 1 μc per microgram was added to varying amounts of IF preparation A and the mixture was administered to test animals. Besides IF preparations, human gastric juices and other materials with vitamin B_{12} binding capacity, such as sodium salt of heparin,[6] chitin sulfate,[6] freshly obtained hog gall bladder bile,[7] human saliva,[8] and saline extract of rat stomach mucosa, were also tested.

C. Test Procedures for Hepatic Uptake.—Test solution (0.6 to 1.0 ml.) containing 40 mμg Co^{60}-labeled vitamin B_{12} with or without test material was injected into the tail vein of a rat within 10 seconds without anesthesia. The intravenous injection had to be accurate and rapid. After varying time intervals from the injection, the animals were anesthetized with ether and the abdomen was opened. The portal vein and hepatic artery were cut simultaneously, followed by the inferior vena cava and the thoracic aorta, respectively. With this sequence of exsanguination, the hepatic blood circulation could be timed accurately. The organs were removed, blotted with filter paper, placed in a 100 ml. bottle to which 20 ml. of 30 per cent potassium hydroxide was added to solubilize the

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*If admixed with 15 μg B_{12}.

tissue. The volume of this solution was brought up to 50 ml. with distilled water and the radioactivity was measured in a well-type scintillation counter. The digestive tract was opened lengthwise and washed thoroughly before alkali digestion. For the measurement of bone and bone marrow radioactivity, the right femur was removed by disarticulation, trimmed, and dissolved in 10 ml. of concentrated nitric acid solution.

D. The Excretion of Radioactivity Following Intravenous Injection.—In some instances, urine and feces were collected following the injection of the test materials. The technique used was that described by Wider and associates.[9] Urine which was absorbed and dried on filter paper was ashed and suspended in 100 ml. bottles with 25 per cent hydrochloric acid; feces were collected and homogenized in the bottles with water and sulfuric acid.

E. The Measurement of Vitamin B_{12} Binding Capacity.—For the measurement of vitamin B_{12} binding capacity, 1 ml. of a normal saline solution of Co^{60}-labeled vitamin B_{12} and an equal volume of IF solution were mixed. The relative amounts of Co^{60}-labeled vitamin B_{12} to intrinsic factor was 1,000 to 2,000 mμg per U.S.P. unit. This ratio provided a surplus of vitamin B_{12} in excess of that necessary for binding. The resulting mixture was dialyzed in Visking cellophane bags against 8 times the volume of a normal saline solution at 4° C. for a period of 24 to 48 hours, under constant shaking to facilitate an establishment of equilibrium. The radioactivity of the diffusate and dialyzate was then measured. From these measurements the B_{12} binding capacity was estimated. In exhaustive dialysis, 2 liters of outside solution were changed at least 4 times. The resting cell technique of Davis[10] was also employed in order to check the measurements of capacity of binding, and the results obtained with both techniques agreed within 10 per cent.

RESULTS

A. Hepatic Uptake and Tissue Distribution.—In experiment 1, three groups of rats (3 in each group) were given intravenously 40 mμg of radioactive vitamin B_{12} together with 0, 1, and 5 mg. of intrinsic factor preparation A per animal. The radioactivity in various organs of rats sacrificed 22 hours after intravenous injection is tabulated In Table I. It can be noted that the addition of IF resulted in greatly increased hepatic uptake with concomitant decrease in the radioactivity in other tissues measured.

TABLE I. TISSUE DISTRIBUTION OF RADIOACTIVE B_{12}*

TISSUE	PER CENT OF INJECTED RADIOACTIVITY (AVERAGE OF 3 RATS)		
	CONTROL†	IF (1 MG.)	IF (5 MG.)
Liver	8.3 (7.5 -9.8)	35.0 (30.0 -44.8)	38.0 (35.2 -41.3)
Kidneys	7.5 (7.0 -7.8)	3.7 (3.2 - 4.2)	4.0 (3.7 - 4.5)
Intestine	8.8 (8.5 -9.8)	4.7 (4.2 - 5.0)	5.0 (4.7 - 5.3)
Stomach	1.50 (1.28-1.65)	0.80 (0.47- 1.05)	0.78 (0.65- 0.97)
Muscle (10 Gm.)	4.0 (3.7 -4.8)	2.0 (1.2 - 2.7)	1.5 (0.7 - 2.0)
Blood (10 ml.)	1.10	0.70	0.65
Brain	0.60	0.32	0.27
Adrenals	0.18	0.05	0.12
Bone	0.23	0.10	0.07
Pancreas	1.58	0.47	
Spleen	1.23	0.52	
Lungs	0.85	0.37	

Parentheses denote the range of values.
*Twenty-two hours after intravenous injection of 40 mμg Co^{60}-B_{12} with and without IF.
†Co^{60}-B_{12} alone.

B. Routes of Administration.—In experiment 2, the routes of injection were compared. For this purpose, 2 female rats each were given 40 mμg

of radioactive vitamin B_{12} solution containing 1 mg. of IF preparation A, intravenously, intramuscularly, or intraperitoneally. Twenty-two hours after injection, the animals were sacrificed; their livers, kidneys, and digestive tracts were removed for radioactivity measurement. The results (Table II) demonstrate again the increase in hepatic uptake, which was largest when the intravenous route was used; whereas, intramuscular injection resulted in a marked decrease in organ radioactivity, indicating retarded distribution of the injected B_{12} and IF complex. The radioactivity in the kidneys and digestive tract was lower in all groups receiving IF.

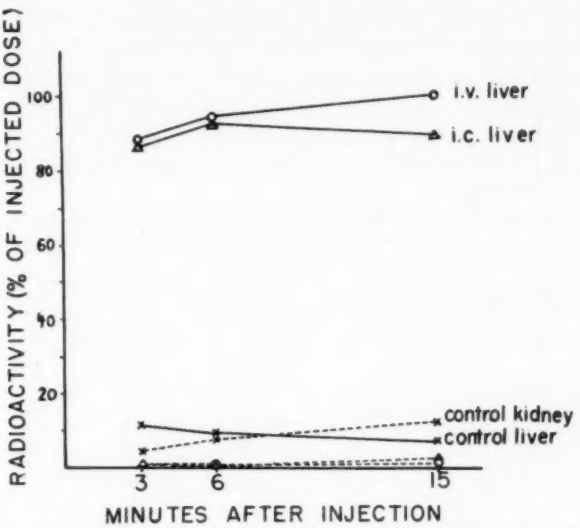

Fig. 1.—Relationship between time of injection and hepatic uptake. One-tenth milligram of IF was combined with 40 mμg Co^{60}-B_{12} and injected by intravenous route (○- - -○) and intracardiac route (△- - -△). Control (X - - -X) received intravenously Co^{60}-B_{12} alone. Solid line denotes liver uptake, and broken line, kidney uptake.

TABLE II. ORGAN UPTAKE OF Co^{60}-B_{12} GIVEN BY VARIOUS PARENTERAL ROUTES

ROUTE	GROUP*	PER CENT OF INJECTED RADIOACTIVITY (AVERAGE OF 2 RATS)		
		LIVER	KIDNEYS	DIGESTIVE TRACT
I.V.	Control	18.2	8.7	12.5
	IF	55.0	3.2	4.7
I.M.	Control	17.1	9.1	11.4
	IF	4.5	1.1	1.4
I.P.	Control	12.5	10.9	12.5
	IF	28.2	4.7	7.6

*Controls received 40 mμg of Co^{60}-B_{12} alone. The IF group received 40 mμg of Co^{60}-B_{12} and 1 mg. IF No. 727.

C. The Relationship Between Time of Injection and Hepatic Uptake.—In order to determine the time required to achieve this increase in hepatic uptake, 40 mμg of Co^{60}-labeled vitamin B_{12} with or without added intrinsic factor was administered to young adult male rats. They were then sacrificed at

3-, 6-, and 15-minute intervals following injection. In addition, intracardiac administration was compared with the tail vein injection, because of the possibility that the speed and sequential order of the intravascular and tissue-to-tissue spread of vitamin B_{12} might give a difference in the uptake pattern. As shown in Fig. 1, the liver uptake was almost maximum in 3 minutes with the vitamin B_{12} remaining in the liver for at least 15 minutes. The slight differences among the figures for 3, 6, and 15 minutes are not significant. The intracardiac route gave essentially the same results. In a striking contrast, the kidney radioactivity in the animals given IF concentrate was negligible during this time, indicating that almost all vitamin B_{12} which was administered with IF concentrate went to the liver. No measurable radioactivity was found in the spleen or lung at 15 minutes.

D. Separate Administration of Vitamin B_{12} and Intrinsic Factor.—To determine whether it is necessary to inject IF and vitamin B_{12} in the premixed state, these two components were injected separately by the intravenous route at different intervals (see Table III). In study I, 1 mg. of IF prep-

TABLE III. SEPARATE INJECTIONS OF IF AND Co^{60}-B_{12}* WITH SHORT INTERVALS

STUDY	FIRST INJECTION	TIME INTERVAL	SECOND INJECTION	AVERAGE LIVER UPTAKE (PER CENT)
I	Co^{60}-B_{12}			11.9
(Male Rats)	Co^{60}-B_{12}	1 hr.	IF (1 mg.)	13.4
	IF (1 mg.)	1 hr.	Co^{60}-B_{12}	12.4
II	Saline	3 min.	Co^{60}-B_{12}	21.3
(Female Rats)	Co^{60}-B_{12} + IF (0.1 mg.)			62.5
	IF (0.1 mg.)	3 min.	Co^{60}-B_{12}	20.0
	IF (5 mg.)	3 min.	Co^{60}-B_{12}	60.8
	Co^{60}-B_{12}	3 min.	IF (0.1 mg.)	21.5
	Co^{60}-B_{12}†	3 min.	IF (5 mg.)	14.0
	IF (0.1 mg.) + 40 mμg nonradioactive B_{12}	3 min.	Co^{60}-B_{12}	22.8
	IF (0.1 mg.) + 40 mμg nonradioactive B_{12}	15 min.	IF (0.1 mg.) + Co^{60}-B_{12}	50.0

Each group had 2 to 3 animals which were sacrificed after 22 hours.

*The Co^{60}-B_{12} was given in 40 mμg doses.

†This group was studied with animals from a different batch because of the restriction imposed by the size of the batch used. The difference of the figure from the control may, therefore, be considered insignificant.

aration A, and/or 40 mμg of Co^{60}-labeled vitamin B_{12} were injected intravenously at 1-hour intervals. The results demonstrated the lack of effect when these 2 components were injected separately at such a time interval. In study II, the time interval was shortened and the amount of IF was reduced to only 0.10 mg. Vitamin B_{12} and/or IF were given intravenously, and the other components were given 3 minutes afterward. The results given in Table III demonstrate that the increase of hepatic uptake was not affected when IF and vitamin B_{12} were given 3 minutes apart. On the other hand, when the IF was increased to 5 mg., an effect was observed, indicating that there was sufficient amount of IF still available to cause this effect. It is interesting to point out that the administration of a premixed solution of 0.1

mg. of IF and 40 mμg of the nonradioactive vitamin B_{12} 15 minutes prior to the administration of a mixture of IF and radioactive vitamin B_{12} did not in any manner interfere with the increase of hepatic uptake. These results, therefore, demonstrate the essentiality to premix the two components and, also, that the capacity of the liver to receive the benefit of intrinsic factor-B_{12} complex had not been reached when 40 mμg of B_{12} and 0.1 mg. of IF were injected.

E. Quantitative Relationship Between Vitamin B_{12} and Intrinsic Factor.—Efforts were made to determine whether a quantitative relationship exists between vitamin B_{12} and a given preparation of IF in the ability of the complex to enhance the hepatic uptake. The study was conducted in 2 ways: (1) by injecting solutions containing a constant amount of radioactive vitamin B_{12} (40 mμg) but varying amounts of IF preparation, and, (2) by injecting solutions containing definite dosage of IF (0.1 and 2 mg.) but varying amounts of radioactive vitamin B_{12}. The results of such a study are presented in Figs. 2 and 3. Fig. 2 shows that at a constant amount of radioactive vitamin B_{12} of 40 mμg, the increase in hepatic uptake at 15 minutes

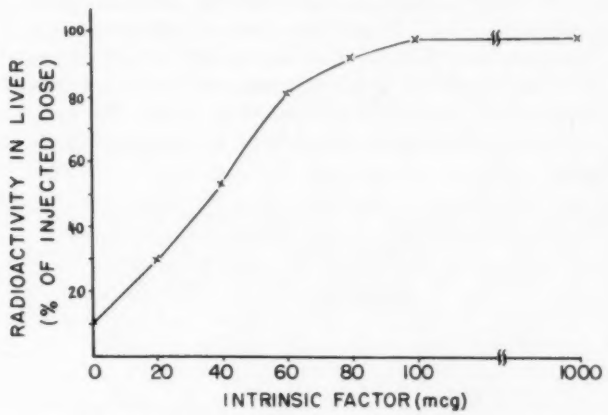

Fig. 2.—Relationship of IF dose to hepatic uptake of B_{12}. Varying doses of IF were mixed with 40 mμg Co^{60}-B_{12} and injected intravenously.

shows a quantitative relationship with the amount of IF added. A plateau, however, is reached when 80 to 100 μg of IF was used. Further increase up to 1 and 5 mg. did not increase the hepatic uptake. It should be pointed out further that 100 μg of IF actually caused a maximum hepatic uptake of 100 per cent. In the second procedure, where the dosages of IF were kept constant at 0.1 and 2 mg. levels, it can be seen that the percentage of hepatic uptake decreases with increasing dosage of vitamin B_{12}. This is most obvious at the lower dosage of IF. At the higher dosage of IF, the uptake remained 100 per cent when as much as 1,000 mμg of vitamin B_{12} was added.

The percentage dropped as the vitamin B_{12} was increased. The absolute quantity of vitamin B_{12} deposited in the liver increased with increasing amounts of vitamin B_{12} administered.

F. Vitamin B_{12} Binding Capacity of Intrinsic Factor Preparation.—The results of the preceding experiments clearly suggested a quantitative relationship between the hepatic uptake and the amounts of IF preparation A when B_{12} was available in excess, whereas B_{12} was almost completely taken up by the liver if the B_{12}-IF ratio were less than approximately 50 mμg: 0.1 mg. or 1:2,000 on a weight basis. Hence, a possibility emerged that the B_{12} binding might be the determining factor in this reaction, and, therefore, the measurement of B_{12} binding capacity of IF preparation A seemed imperative. To this end, 100 to 500 mμg of Co^{60}-B_{12} was combined with varying levels of preparation A and dialyzed against 8 times the volume of saline for 24 hours. With this equilibrium dialysis, and at the levels of 0.1 to 0.5 mg. of intrinsic factor, the binding power was estimated as 50 to 65 mμg per 0.1 mg. which is close to the ratio of 1:2,000 obtained from the in vivo studies. When excess amounts of nonradioactive B_{12} were added to such IF-Co^{60}-B_{12} mixtures and dialyzed under the same condition, much lower radioactivities were measured inside the bags, suggesting an exchange between bound Co^{60}-B_{12} and added nonradioactive B_{12}. Exhaustive dialysis also gave the same binding figures. However, the addition of a large excess of IF did not prevent the dissociation of radioactivity which was estimated to be all bound before dialysis, such dissociation usually being 30 to 35 per cent. The values of binding obtained with the resting cell method were in agreement with those obtained by dialysis.

TABLE IV. SEQUENCE OF B_{12} ADDITION TO INTRINSIC FACTOR AND LIVER UPTAKE OF B_{12}

AMOUNT OF B_{12}	ORDER OF MIXING			CONTROL	
	IF + Co^{60}-B_{12} + B_{12}	IF + B_{12} + Co^{60}-B_{12}	B_{12} + Co^{60}-B_{12} + IF	B_{12} + Co^{60}-B_{12}	IF + 40 mμg Co^{60}-B_{12}
Co^{60}-B_{12} = 40 mμg B_{12} = 460 mμg	39.0 (97%)	2.5	6.8	1.5	38 (95%)
Co^{60}-B_{12} = 40 mμg B_{12} = 960 mμg	35.6 (89%)	1.4	3.9	0.3	

Figures are millimicrograms of Co^{60}-B_{12} recovered from the liver.
Parentheses denote that materials were mixed and incubated first.

G. B_{12} Binding and Preferential Liver Uptake of B_{12}.—The finding that the maximum liver uptake occurred at a certain ratio of B_{12} to IF concentrate close to that in the binding experiment prompted us to study the essentiality of binding in the increase of hepatic uptake. The following series of experiments were performed. First, 40 mμg of Co^{60}-B_{12} was incubated with 0.1 mg. of IF 727 at 37° C. for 5 minutes. Our previous experiments showed that the radioactivity is completely bound. Then 460 and 960 mμg of nonradioactive B_{12} were added to the incubated mixture, making the total B_{12} 500 and 1,000 mμg, respectively, and immediately injected intravenously into the rats. The addition of the nonradioactive B_{12} does not replace the bound radioactive vitamin B_{12}. The animals were sacrificed after

15 minutes, and the radioactivity in the liver was measured. In spite of the addition of as much as 12.5 and 25 fold nonradioactive B_{12}, 89 to 97 per cent of the added radioactivity was recovered from the liver (Table IV). When the IF preparation was mixed with the same amount of nonradioactive B_{12}, and 40 mμg Co^{60}-B_{12} was added subsequently to the IF concentrate, the injection of such a mixture resulted in little increase in the hepatic uptake which was probably due to simple isotope dilution. This clear-cut difference in liver uptake, as a result of the change of adding order, indicates that the radioactivity of the B_{12} added to IF concentrate was not diluted with the subsequently added nonradioactive B_{12}. These results are highly suggestive that IF concentrate and B_{12} form a complex first and that the liver has a preferential access to such IF concentrate bound B_{12} in vivo.

H. Effects of Substances Other Than Intrinsic Factor Concentrate.—Besides IF, several biologic materials with B_{12} binding property were also tested to determine whether they will also enhance the hepatic uptake of radioactive B_{12}. Four separate experiments were conducted with different materials tested as indicated in Table V. In the first experiment (Study A), saline solutions of chitin sulfate, untreated hog bladder bile, and IF preparation A were given intravenously to rats. Those receiving an isotonic salt solution served as controls. The injection of bile was conducted slowly to avoid shock. Chitin sulfate failed to show any effect while the hog bile had a definite but less marked effect on the liver B_{12} uptake than the IF concentrate. When the dose of bile was raised to 0.6 ml. (Study B), the hepatic uptake of B_{12} was definitely elevated over the control. In addition, the fecal excretion of radioactivity in bile treated animals was remarkably increased. The administration of 0.1 mg. of IF preparation B with a lower IF activity on a weight basis together with 40 mμg Co^{60}-B_{12} increased the hepatic uptake slightly while a dose of 1.0 mg. caused a significant increase in liver uptake, although not to the same level as that obtained with the more potent preparation A (Study C). The binding capacity of preparation B was measured to be about 18 mμg per milligram. In the fourth experiment (Study D), the animals were killed after 15 minutes, a time at which the effect of the IF concentrate was believed to be maximum. The rat gastric mucosa extract (which had IF activity, as tested with the intestinal loop technique[11]), hog bile, human saliva, and gastric juice showed definite enhancement of liver B_{12} uptake.

I. Heat Inactivation of Intrinsic Factor Concentrate.—Preparation 727 was dissolved in 0.1 M phosphate buffer of pH 7.2 at the concentration of 1 mg. per milliliter and heated in vigorously boiling water at 100° C. for 30 minutes. After cooling, an equal volume of Co^{60}-B_{12} was added to the mixture. No inactivation was obtained in terms of liver B_{12} uptake. When the same IF concentrate was dissolved in a normal saline solution, pH adjusted to 2.0 with hydrochloric acid heated at 100° C. for 30 minutes, and then neutralized, the hepatic uptake was reduced by 60 per cent. The binding capacity was also reduced to the same extent.

DISCUSSION

Our data clearly demonstrate that parenteral administration of IF concentrate and Co^{60}-B_{12} mixture to rats enhanced the hepatic uptake of Co^{60}-B_{12} and that this phenomenon was completed within 3 to 6 minutes. The tested preparation A, having a very high intrinsic factor activity, was found to be capable of permitting the liver to take up the simultaneously injected B_{12} almost totally, provided that the B_{12}-IF concentrate ratio was approximately 1:2,000 on weight basis or smaller. The liver seems to have a tremendous capacity of taking up B_{12} under such conditions, as indicated by Fig. 3. While Herbert[4] observed a decrease in B_{12} uptake by the liver at high IF concentrations, no such diminution of uptake was obtained in vivo with increasing amounts of preparation A up to 5 mg. It may be that preparation A is so purified as to contain no inhibitory components.

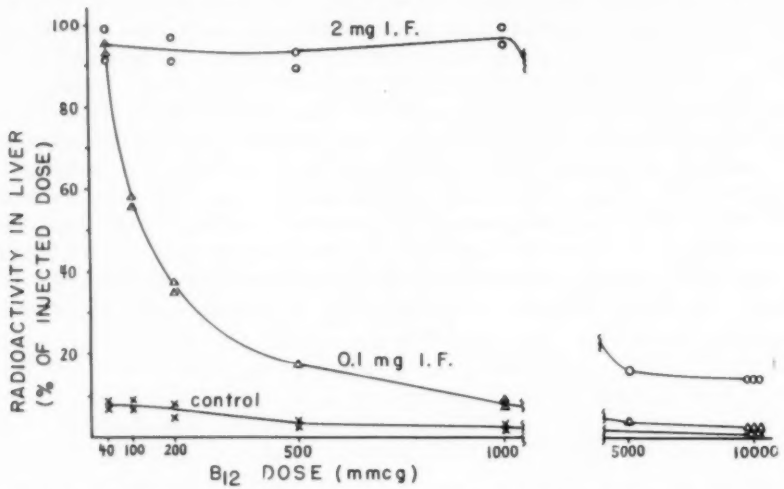

Fig. 3.—Relationship between amount of Co^{60}-B_{12} and hepatic uptake. Varying doses of Co^{60}-B_{12} were mixed with 2 mg. (○ - - - ○) and 0.1 mg. (△ - - - △) of IF and injected. Control (X - - - X) received Co^{60}-B_{12} alone.

Although it required a larger amount of the less active IF preparation B to cause the same phenomenon, it is not the purpose of this paper to correlate IF potency with the hepatic uptake activity. It is doubtful that this relationship exists since bile and saliva with no demonstrated IF activity likewise facilitate hepatic uptake. It should be mentioned that both these substances have strong B_{12} binding capacity.

Much discussion has been made as to the importance of vitamin B_{12} binding capacity relative to IF activity. We have maintained that B_{12} binding is an integral property of the IF molecule. In this study, it has been shown very clearly that in order for B_{12} to be taken up by the liver, B_{12} has to be mixed with IF concentrate or certain other B_{12} binders before injection, and that such B_{12} binders permit the liver to pick up only the B_{12} which was

bound. This concept receives support from the finding that the binding ratio of B_{12} and IF is in close agreement with the ratio of the two components in the mixture which yielded the most efficient liver uptake. However, it is apparent that there are different mechanisms of B_{12} binding and that only certain types of binding or B_{12}-protein complex are responsible for this phenomenon, since both chitin and heparin which also bind B_{12}[6] were incapable of increasing the hepatic uptake of B_{12} (Table V). Whether such a bound B_{12} is released from IF before entering the liver is not known, but

TABLE V. COMPARISON OF EFFECT BY INTRINSIC FACTOR AND OTHER BIOLOGIC SUBSTANCES ON B_{12} UPTAKE

STUDY	TIME OF SACRIFICE	MATERIAL	PER CENT RECOVERED RADIOACTIVITY			
			LIVER	KIDNEYS	DIGESTIVE TRACT	FECES
A	22 hr.	Control*(3)	18.3	8.7	12.5	
		IF, 1 mg.(2)	55.0	3.2	4.7	
		Chitin-SO_4, 0.8 mg.(2)	18.7	9.5	13.3	
		Hog bile, 0.4 ml.(2)	28.0	2.7	3.2	
B	22 hr.	Control*(3)	10.5	9.5	13.8	0.2
		IF, 0.1 mg.(2)	58.8	3.2	4.7	5.3
		Bile, 0.6 ml.(2)	37.8	3.0	3.0	23.0
C	22 hr.	Control*(3)	16.7	15.0	14.8	
		IF, 0.1 mg.(2)	56.0	5.2	5.7	
		IF, 1 mg.(2)	60.5	6.2	4.7	
		IF No. 519, 0.1 mg.(2)	19.5	15.2	12.5	
		IF No. 519, 1 mg.(2)	37.2	11.7	8.2	
D	15 min.	Control*(3)	9.2			
		IF, 0.1 mg.(2)	90.0			
		Rat gastric mucosa extract(2)	50.5			
		Bile, 0.4 ml.(2)	66.0			
		Human saliva(2)	51.0			
		Human gastric† juice, 0.4 ml.(2)	30.8			
		Heparin sodium 4 mg.(2)	8.5			

The number in parentheses indicates the number of rats.
In Study B, male rats were used; in all other studies, females were used.
*Forty millimicrograms of Co^{60}-B_{12} alone; intrinsic factor 727 was used.
†Contained free HCl of 20 units, neutralized before use.

our unpublished data indicate that it is not the case. The fact that a saline extract of the rat gastric mucosa had the same effect makes it rather unlikely that this is merely a phenomenon given by heterologous proteins. Although the liver has been reported[12] to behave similarly to injected antigens, the lung and the spleen, the latter in particular, take them up more efficiently than the liver. In this study, neither the spleen nor the lung was found to pick up measurable amounts of B_{12}-IF complex to compete with the total uptake by the liver. The data presented in Table II demonstrating that the IF concentrate and B_{12} complex did not readily go to the liver or other organs when injected intramuscularly strongly suggest the lack of diffusibility of the IF concentrate B_{12} complex from the site of injection. Contrary to Miller's report[2] on the presence of receptor proteins in various tissues in vitro, this preferential uptake seems to be limited to the liver in the in vivo system.

It is convenient to speculate in the light of these findings that IF or some kind of macromolecular substance circulates in the blood as a B_{12} carrier to transport B_{12} from the intestine and other organs to the liver. At this time, it is difficult to correlate this phenomenon of preferential liver uptake following intravenous administration of IF concentrate with the role of IF in B_{12} absorption in the intestinal canal. Whether the reaction observed under our conditions is explained by the reported in vitro reactions[1-5] awaits further investigations.

SUMMARY

IF concentrates and other biologic substances have been studied for their effect upon liver uptake of radioactive vitamin B_{12} in vivo in the rat. When a highly purified intrinsic factor preparation was mixed with Co^{60}-B_{12} in certain ratios and injected intravenously, a complete uptake of Co^{60}-B_{12} by the liver was observed within a few minutes. A crude intrinsic factor preparation, an extract of rat stomach mucosa, hog gall bladder bile, and human saliva and gastric juice, showed similar effect, but to a lesser extent. It has also been demonstrated that the portion of B_{12} which is bound to intrinsic factor concentrate is preferentially taken up by the liver. The possible physiologic significance of such a phenomenon has been discussed.

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EFFECT OF LIVER DAMAGE AND REGENERATION ON VITAMIN B_{12} CONCENTRATION IN RAT LIVER

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IN A previous report[1] it was shown that acute and massive liver necrosis produced by intraportal carbon tetrachloride injection into dogs was followed by an immediate rise of serum vitamin B_{12} levels. These experiments demonstrated that the excess of vitamin B_{12} in serum was due to its liberation from damaged liver cells. They also indicated that the high serum vitamin B_{12} levels found in acute and chronic liver disease in man[2-4] might be the result of hepatocellular necrosis.

The questions concerning the fate of the released vitamin B_{12} and whether an injured liver is able to take up vitamin B_{12} remained unanswered. In the present paper an attempt was made to elucidate some of these problems.

MATERIALS AND METHODS

Male albino rats of the Hebrew University strain, weighing 120 to 130 Gm. and kept on a stock diet, were used. All manipulations on the rats, described below, were done during ether anesthesia. Laparotomy was performed on 31 rats, and 0.02 ml. of CCl_4 in olive oil (1:1) was injected into a small mesenteric vein (intraportal injection). At the end of the experimental periods, which ranged for 2 to 24 hours, 4 groups of animals were sacrificed, 2, 6, 12, and 24 hours after injection, by bleeding from the aorta. The blood and livers of each group of animals were pooled for assay of vitamin B_{12}. Eight animals not injected with CCl_4 served as controls.

In another study, necrotic damage was produced in two groups of 6 rats, by a subcutaneous injection of CCl_4 in oil (3:2) (0.3 ml. CCl_4 per 100 Gm. body weight). One-tenth microcurie $B_{12}Co^{60}$ was injected into the tail vein 6 or 24 hours later. Twelve control rats of the same weight were given an injection of olive oil prior to $B_{12}Co^{60}$ injection. All animals were sacrificed 48 hours after the $B_{12}Co^{60}$ injection.

Partial hepatectomy was done on 34 rats according to the method of Higgins and Anderson.[5] $B_{12}Co^{60}$ (0.1 μc) was injected intravenously 2 days after the operation. The control group consisted of 24 sham-operated and 42 intact rats of similar weight. All animals were sacrificed 24 hours to 12 days following $B_{12}Co^{60}$ injection.

Sections removed for histologic examination were fixed in Carnoy's fluid, cut at 6 μ and stained with hematoxylin and eosin.

Preparation of Tissue Homogenates.—The determination of vitamin B_{12} in organs was made on tissue homogenates prepared in water, either in a Waring Blendor or in a Potter Elvejhem homogenizer. Unless examined immediately the homogenates were frozen and stored at −20° C.

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Microbiologic Assay.—Vitamin B_{12} was determined microbiologically by *Escherichia coli* assay.[6] Total serum B_{12} was measured after liberation of the vitamin from the serum protein after acid precipitation.[7] Tissue B_{12} content was determined in samples of the homogenates. One gram of homogenate was added to 100 ml. of 0.001 N acetate buffer (pH 4.5) and autoclaved at 110° C. for 30 minutes in the presence of 1.0 mg. of potassium cyanide. After centrifugation the supernatant was neutralized and assayed. The final value is the mean of 3 determinations of each sample.

Vitamin $B_{12}Co^{60}$ Determination.*—The vitamin $B_{12}Co^{60}$ (specific activity 0.83 $\mu c/\mu g$) was diluted with water 1:50. Aliquots of this solution were used as standards for counting radioactivity in the homogenates and for microbiologic assays. The determinations were made on 10 ml. samples in a liquid scintillation counter with annular geometry. The time of the counting was continued sufficiently to give a counting error of less than 3 per cent. The $B_{12}Co^{60}$ uptake by the tissues was expressed in counts per minute per gram of wet tissue.

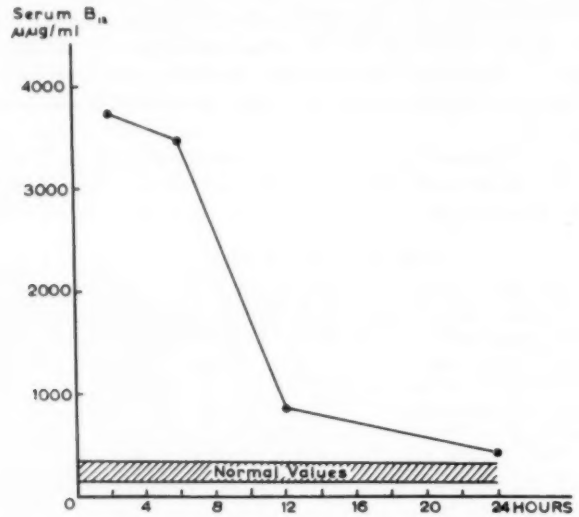

Fig. 1.—Serum vitamin B_{12} levels after intraportal CCl_4 injection in rats. Each point represents the mean value of 6 to 8 rats.

TABLE I. EFFECT OF INTRAPORTAL CCl_4 ON VITAMIN B_{12} CONTENT OF POOLED RAT LIVER

HOURS AFTER CCl_4 INJECTION	NUMBER OF RATS	B_{12} μg/GM. LIVER
2	8	28
6	7	22
12	11	49
24	5	43
Controls	8	57

RESULTS

Effect of Intraportal CCl_4.—Acute liver injury in rats induced by CCl_4 caused a rapid rise in serum B_{12} levels within 2 to 6 hours following the injection.

*Kindly donated by Merck Sharp & Dohme (Division of Merck & Company, Inc.), Rahway, N. J.

The B_{12} serum level fell steeply during the subsequent 12 to 24 hours (Fig. 1). Concomitant with the rise in serum B_{12} levels there was a fall in the total B_{12} content of the liver 2 and 6 hours following the acute injury. After 24 hours there was a marked increase of the B_{12} content of the liver but the vitamin did not reach its control level (Table I).

$B_{12}Co^{60}$ Uptake by Injured Liver.—The observation that the liver rapidly tends to regain its normal B_{12} concentration after acute injury suggested that the injured liver is capable of concentrating the vitamin. This possibility was investigated by determining the uptake of $B_{12}Co^{60}$ in livers damaged by subcutaneous CCl_4 injection. This route of administration was chosen since the injury produced by it is diffuse and more closely resembles acute hepatitis of man than that produced by intraportal injection.

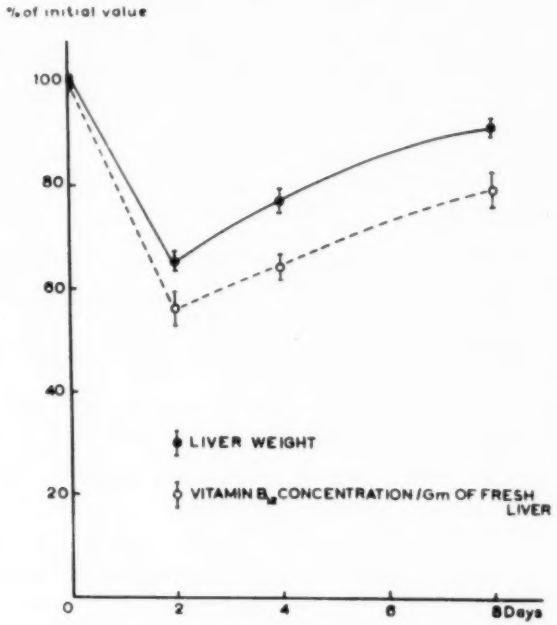

Fig. 2.—Restoration of liver vitamin B_{12} content and weight following subtotal hepatectomy in rats. Each point represents 4 to 5 rats ± S.E. The initial weight of the liver was calculated from the weight of the removed part which was 66 ± 4 per cent of total, as found in a series of 30 control rats.

The results given in Table II show an increase in the $B_{12}Co^{60}$ uptake by the liver of rats treated with CCl_4 as compared to controls when measured 48 hours after $B_{12}Co^{60}$ injection. Similar results were obtained when $B_{12}Co^{60}$ was administered 6 hours after CCl_4 and the uptake measured 48 hours later. No correction is given for the blood content of the liver, since the level of radioactivity of blood at the time of determination was found to be negligibly low.

Histologic findings in the liver were similar to those reported by others,[8] a combination of necrosis, fatty and hydropic change, and active regeneration.

TABLE II. EFFECT OF CCl_4 ON THE HEPATIC UPTAKE OF $B_{12}Co^{60}$ IN RATS*

CONTROL (0.5 ML. OIL/100 GM.)			CCl_4 (0.5 ML. 100 GM.)		
RAT NO.	LIVER WEIGHT/GM.	C.P.M./GM.	RAT NO.	LIVER WEIGHT/GM.	C.P.M./GM.
1	5.0	90	7	5.7	172
2	5.3	85	8	5.0	133
3	5.5	81	9	5.2	180
4	5.0	99	10	5.3	126
5	5.5	105	11	5.5	180
6	4.5	96	12	4.6	115
Mean ± S.E.		92.6 ± 3.7			151 ± 12.0
p			$p < 0.001$		

*One-tenth microcurie $B_{12}Co^{60}$ was injected intravenously 24 hours after CCl_4 administration. The measurements were done 48 hours after $B_{12}Co^{60}$ administration.

Uptake of $B_{12}Co^{60}$ After Partial Hepatectomy.—The uptake of $B_{12}Co^{60}$ by regenerating rat liver was studied on partially hepatectomized animals sacrificed at different time intervals following $B_{12}Co^{60}$ injection. From the fourth day following hepatectomy, i.e., two days following $B_{12}Co^{60}$ injection, there was a statistically significant increase in the B_{12} uptake by the hepatectomized rats as compared to sham-operated and to normal controls (Table III). The sham-operated rats had a slightly higher uptake than the normal controls.

TABLE III. HEPATIC UPTAKE OF $B_{12}Co^{60}$ IN NORMAL, SHAM-OPERATED, AND HEPATECTOMIZED RATS*

DAYS AFTER $B_{12}Co^{60}$ INJECTION	COUNTS PER MINUTE PER GRAM LIVER			P†
	NORMAL	SHAM-OPERATED	HEPATECTOMIZED	
1	78 ± 5.21‡ (8)§		122 ± 6.26 (5)	
2	105 ± 4.24 (16)	122 ± 8.54 (10)	161 ± 8.95 (12)	<0.01
7	124 ± 6.17 (11)	127 ± 9.9 (10)	180 ± 17 (10)	<0.02
12	124 ± 6.8 (7)	129 ± 5.5 (4)	176 ± 17.3 (7)	<0.02

*One-tenth microcurie B_{12}-Co^{60} injected 2 days after operation.

†The significance of the difference between the sham-operated and hepatectomized rats was tested with Student's "t" test.

‡Mean and standard error.

§Figures in parentheses represent number of rats.

Restoration of Liver B_{12} After Partial Hepatectomy.—The increased avidity of the regenerating liver for injected B_{12} prompted the investigation of the total B_{12} content of the liver following hepatectomy. It is evident from Fig. 2 that the restoration of the hepatic vitamin B_{12} content to its preoperative value lagged behind the growth of the regenerating organ.

DISCUSSION

Acute liver injury by CCl_4 in the rat resulted in a release of vitamin B_{12} into the blood stream, similar to that reported previously in the dog.[1] The rise in serum B_{12} level occurred very rapidly after the injury but was a rather short-lived one. At 24 hours after injury the values were only slightly higher than normal, while at 2 to 6 hours the increase was more than tenfold.

These results would explain the comparatively slight B_{12} rise in the serum obtained by Yamamoto and associates[9] 24 hours following CCl_4 administration in rats. According to expectation, the B_{12} content of the liver was lowest shortly following CCl_4 injection but tended to regain normal values after 24 hours. A similar loss of hepatic B_{12} in rats, resulting from chronic CCl_4 injury, was reported by Wolff and co-workers.[10] The rapid disappearance of the excess B_{12} from the blood raised the question of the fate of the vitamin. Preliminary experiments with $B_{12}Co^{60}$ in a dog receiving CCl_4 intraportally showed that only a part of the vitamin was excreted in the urine.[11]

It seems plausible that a part of the released vitamin B_{12} returns to the liver. An increased uptake of labeled B_{12} by the liver was observed after CCl_4 damage. It is probable that the vitamin is taken up primarily by the cells outside of the necrotic areas, where active regeneration takes place soon after the injury. However, the exact localization of labeled B_{12} in the injured liver cannot be ascertained further without radioautographic studies.

That the regenerating liver is actually capable of increased $B_{12}Co^{60}$ uptake was demonstrated in the experiments on rats following partial hepatectomy. However, this increased uptake of B_{12} was limited only to the regenerating normal hepatic tissue, and was not observed in hepatic tumors produced by dimethylaminoazobenzene in which a very low $B_{12}Co^{60}$ uptake was found.[12]

The restoration of the normal B_{12} concentration of the liver cell lagged behind the growth of the organ and did not reach its normal level 8 days following the operation. The low B_{12} content of the liver after partial hepatectomy (expressed as micrograms of B_{12} per gram of liver tissue) has been correlated with the lower protein content of regenerating liver.[13] A similar observation has been described for vitamin A.[14]

An increased demand of B_{12} by the regenerating liver is in keeping with the view of the role which vitamin B_{12} has been shown to play in protein synthesis.[15]

SUMMARY

1. Acute liver injury caused in rats by intraportal CCl_4 injection resulted in a rapid rise in serum vitamin B_{12} levels, accompanied by a fall in the B_{12} content of the liver. Twenty-four hours following the injection both serum and liver B_{12} levels tended to regain control values.

2. Following CCl_4 injury there was an increased uptake of $B_{12}Co^{60}$ by the liver.

3. A similar increase in $B_{12}Co^{60}$ uptake was found in regenerating rat liver following partial hepatectomy.

4. The restoration of the B_{12} content of the regenerating liver was shown to lag behind that of the weight of the organ.

We wish to thank Prof. J. Gross for his kind interest and help in the preparation of this manuscript.

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THROMBOLYSIS IN DOGS WITH HUMAN FIBRINOLYSIN

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THE production of segmental radiopaque blood clots has been previously reported by the author.[1] This report illustrates the effectiveness of intravenous fibrinolysin therapy on induced radiopaque thrombi in partially occluded dog veins.

METHODS

Segmental radiopaque blood clots were produced in dog veins by the entrapment of radiopaque material in an induced blood clot. The clot is formed by a method previously described[1] with a mixture of thromboplastin and radiopaque material. One hour after formation of the clot, the treated animals received intravenously human fibrinolysin* dissolved in 25 ml. of 5 per cent dextrose and saline over a period of 3 minutes. The fate of the clot was observed by serial x-ray examinations in both the treated and control animals. Prothrombin time and two-stage prothrombin determinations were performed by established methods.[2] Fibrinogen was determined by the biuret method on the isolated plasma clot. Esterase activity was determined by the synthetic substrate method of Sherry, using benzoyl-arginine-methyl ester (BAMe)[3]. Clot lysis was determined by the method of Loomis[4] at 37° C. Platelet (direct)[5] and white blood counts were determined by standard methods.

RESULTS

In Vivo Clot Formation and Clot Lysis.—Fig. 1 shows the x-ray of a typical clot produced in a dog vein. The x-ray shown in Fig. 1, *A*, was made 60 minutes after the injection of the thromboplastin-radiopaque mixture. The x-ray in Fig. 1, *B*, was made 48 hours after the injection. The x-ray shows some shrinkage of the radiopaque clot over the 48 hour period. In addition, the clot was demonstrated by palpation of the vein.

Fig. 2, *A*, shows a clot produced in another dog 1 hour after the injection of the thromboplastin-radiopaque material and just prior to the intravenous injection of fibrinolysin in another vein. Fig. 2, *B*, was made 1 hour after the injection of fibrinolysin and Fig. 2, *C*, was made 22 hours later. It is apparent from these x-rays that there was an almost complete lysis of the radiopaque

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*Courtesy of Drs. R. V. Chapple and H. O. Singher, Ortho Research Foundation, Raritan, N. J. The fibrinolysin is purified from human plasma fraction III, activated by purified streptokinase, lyophilized, and stored at −20° C. until used. The units are based on an empirical in vitro assay method.

clot within the 24 hour period after the injection of fibrinolysin. There were no instances of re-formation of the clots in the dogs receiving fibrinolysin when examined at necropsy 3 days later.

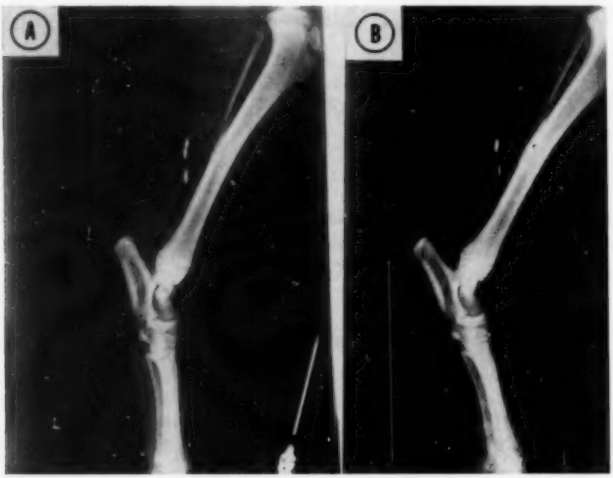

Fig. 1.—X-rays of radiopaque blood clot produced in normal dog vein. *A*, One hour after clot formation. *B*, Forty-eight hours after clot formation.

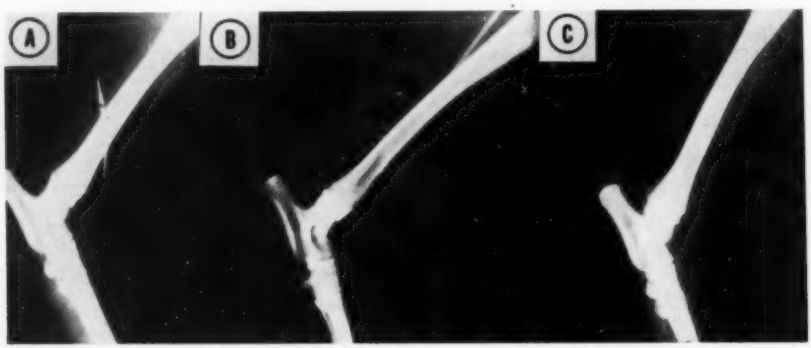

Fig. 2.—X-rays of radiopaque blood clot in normal dog vein. *A*, One hour after clot formation, and prior to the systemic injection of fibrinolysin. *B*, One hour after the injection of fibrinolysin. *C*, Twenty-four hours after the injection of fibrinolysin.

Systemic Effect of Intravascular Clot Formation and Lysis.—The effects of the intravenous injection of fibrinolysin on the various coagulation factors was observed. A significant reduction in fibrinogen after the injection of fibrinolysin was noted (Fig. 3). Serial studies have shown this reduction of fibrinogen to be a temporary phenomenon lasting 8 hours in the average dog.

Fig. 4 illustrates the average rise in the esterase activity of the general circulation as measured by the BAMe method. The dogs injected with fibrinolysin revealed an average rise of 15 units per milliliter, whereas the control

dogs showed a rise of only 4 units per milliliter. Serial observations have shown that the duration of increased esterase activity is variable but usually lasts 2 hours. Lysis times as determined by the Loomis method[4] at 37° C. showed no change with serial determinations in either the control or treated animals.

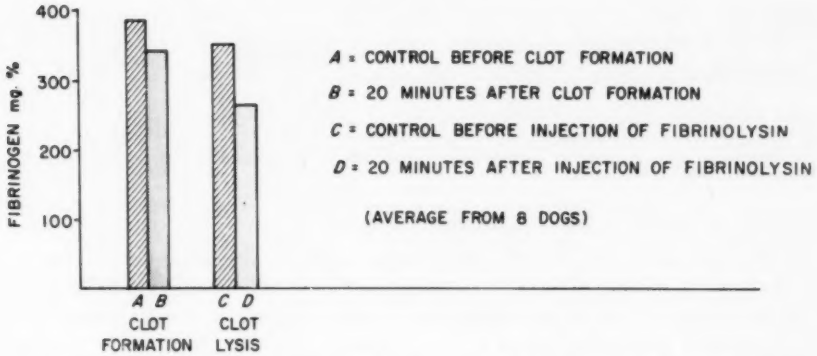

Fig. 3.—Effects of intravascular clot formation and clot lysis on systemic fibrinogen level. Bar graph illustrating the average decrease in fibrinogen in the plasma of the control and dogs treated with intravenous fibrinolysin.

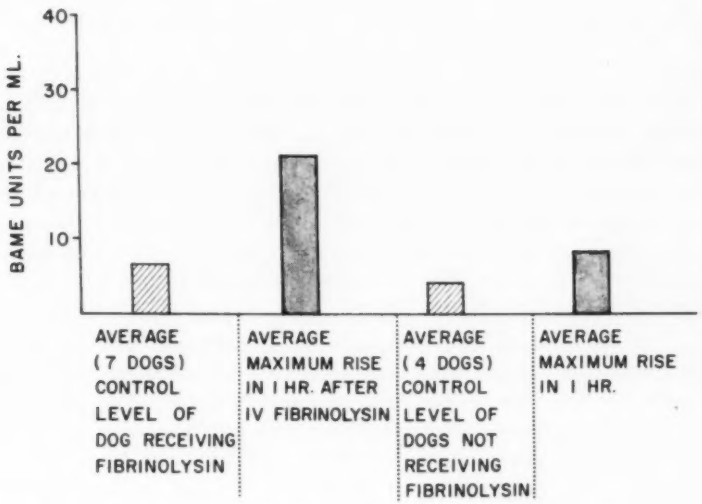
40
30
20
10
BAME UNITS PER ML.
AVERAGE (7 DOGS) CONTROL LEVEL OF DOG RECEIVING FIBRINOLYSIN
AVERAGE MAXIMUM RISE IN 1 HR. AFTER IV FIBRINOLYSIN
AVERAGE (4 DOGS) CONTROL LEVEL OF DOGS NOT RECEIVING FIBRINOLYSIN
AVERAGE MAXIMUM RISE IN 1 HR.

Fig. 4.—Effect of fibrinolysin on BAMe assay levels. Bar graph illustrating the average rise in one hour in BAMe units per milliliter of plasma in the control and dogs treated following systemic fibrinolysin administration.

Necropsy Data.—Table 1 summarizes the necropsy data of the dissected veins in the series of 15 dogs. Six of the 7 control dogs, and 1 dog receiving a very small dose of fibrinolysin, had clots completely occluding the veins 5 days after the induction of the clot. In contrast, 7 dogs treated with 200,000

units of fibrinolysin had no evidence of clots in their veins 3 days after the induction of the clot. Gross examination of the dissected animals' veins revealed no hemorrhagic reaction in either the treated or the nontreated animals.

TABLE I. EFFECTS OF SYSTEMIC FIBRINOLYSIN ADMINISTRATION ON INDUCED RADIOPAQUE BLOOD CLOTS

NO. DOGS	UNITS OF FIBRINOLYSIN INJECTED	AVERAGE AGE OF CLOT AT NECROPSY	NO OF VEINS COMPLETELY OCCLUDED AT NECROPSY*
7	0	5 days	6
1	5,000	6 days	1
7	200,000	3 days	0

*Occluded 100 per cent as determined by the failure of injected saline to move past the clot in the intact vein.

DISCUSSION

On the basis of animal experiments with radiopaque clots, human fibrinolysin is an effective in vivo thrombolytic agent. Doses of fibrinolysin that significantly accelerate the lysis of blood clots did not alter the level of circulating coagulation proteins or produce significant decrease in in vitro lysis time. The data on increase in in vivo lysis of "acute" experimental blood clots with fibrinolysin are in agreement with previous reports.[6-10] Previous workers[7-10] have produced experimental thrombi by various agents such as thrombin, serum fractions or radioactive bovine fibrinogen, which are contaminated with profibrinolysin; thus casting some doubt on both in vitro and in vivo studies in these reports. The radiopaque thrombi in this study were produced with brain thromboplastin which was shown by in vitro studies to be free of both profibrinolysin and fibrinolysin. Esterase activity as measured by the synthetic substrate method was increased. This increase in esterase activity can be associated with increased levels of first component of complement, thrombin, or fibrinolysin. In the studies reported in this paper it is assumed that the increased esterase activity is related to an increase in fibrinolysin activity. However, this was not associated with acceleration in the lysis time of the in vitro fibrin clots.

Increase in the lysis of the radiopaque blood clots and decrease in the circulating level of fibrinogen after fibrinolysin injection suggest that a temporary increase in the circulating level of fibrinolysin can be achieved by intravenous administration. The accelerated lysis of the in vivo clots illustrated in this report represents a direct fibrinolytic effect of fibrinolysin on the induced clot. The failure to demonstrate any marked changes in the in vitro lysis time demonstrates the limitation of in vitro methods for measuring fibrinolytic activity. This would suggest that effective in vivo lysis of thrombi can be induced without alteration in the various coagulation factors, thereby increasing the potential usefulness of fibrinolysin. It is important to note that no hemorrhagic or clot re-formation occurred in the treated animals of this study.

SUMMARY

The intravenous administration of concentrated human fibrinolysin is an effective method of accelerating the lysis of induced radiopaque blood clots in partially occluded dog veins.

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THE MECHANISM OF REMOVAL OF ROSE BENGAL FROM THE PLASMA OF THE RAT

Determined by Autoradiography and Radioassay

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INTEREST in the use of rose bengal in the study of liver function has recently been revived. The dye, which has a rose color, was used for this purpose as early as 1923.[1] It was found to be suitable for colorimetric measurements in plasma,[2] and was believed to be removed from the circulation specifically by the liver.[3, 4] The determination of its rate of disappearance represented a measure of liver function. The compound readily lends itself to labeling with radioiodine, its chemical structure being tetrachlortetraiodofluorscein. Recently, Taplin and associates[5] introduced the use of an I^{131}-labeled form of this material (to be abbreviated as RBI^{131}), which allowed measurements based on the radioactivity of the isotope and made possible the use of much smaller amounts of the compound. The interpretation of their results is based upon evidence that the dye is removed by polygonal cells of the liver and not by cells of the reticuloendothelial system. The evidence for specific polygonal cell extraction was derived from experiments involving "blocking" the reticuloendothelial system of rabbits by various means.

In the present study we have attempted to demonstrate the mechanism by which the liver handles the dye, using the technique of autoradiography, with corollary radioassay data on tissue distribution.

MATERIALS AND METHODS

RBI^{131}, 200 to 400 μc (1 to 4 mg.), was injected into the tail veins of each of 45 anesthetized adult male Wistar rats weighing from 200 to 400 grams. Success of the injection was immediately determined by external radiation survey and later by radioassay of the entire tail. Animals having significant amounts of the dye extravasated at the site of injection were discarded. Most of the animals were killed at intervals varying between 2 and 130 minutes; one group was killed after 3 days. At the appropriate time each animal was again anesthetized and a midline abdominal incision was made. The portal vein and later the inferior vena cava were isolated and perfused with physiologic saline, 1 or 2 per cent copper acetate, and 10 per cent formalin in that order; the entire perfusion was accomplished as rapidly as possible. The saline perfusion served to remove most of the blood from the liver; the copper acetate served to fix the I^{131} and to prevent "leaching"† of

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†We have found (unpublished data[6]) that immersion of tissues in copper acetate minimizes loss of some iodine compounds during subsequent histologic processing.

the isotope as the tissue was carried through the subsequent fixing processes. The success of the perfusion could readily be determined; there was blanching of the liver with saline, a change to a blue-gray color due to copper acetate, and, finally, the development of a gray fixed appearance as a result of formalin. At the completion of the perfusion, the animals that were still alive were killed. Radioassays of liver, spleen, and the entire gastrointestinal tract were performed upon all animals except the 3-day group. In addition, radioassays were done on samples of thyroid, muscle, kidney, skin, and spleen from 1 or 2 animals at each time interval to see whether any significant amount of the dye had concentrated at unexpected sites. The radioassays consisted of measurements made in a well scintillation counter upon tissues weighed in the fresh state and homogenized in 10 per cent NaOH. Autoradiograms were prepared from all liver samples and selected samples of spleen and gastrointestinal tract. Routine histologic methods were used and autoradiograms were made with Type A, NTB and stripping film emulsions. A permanent apposition technique[7] was used; thus the illustrations show both the histologic section and the emulsion.

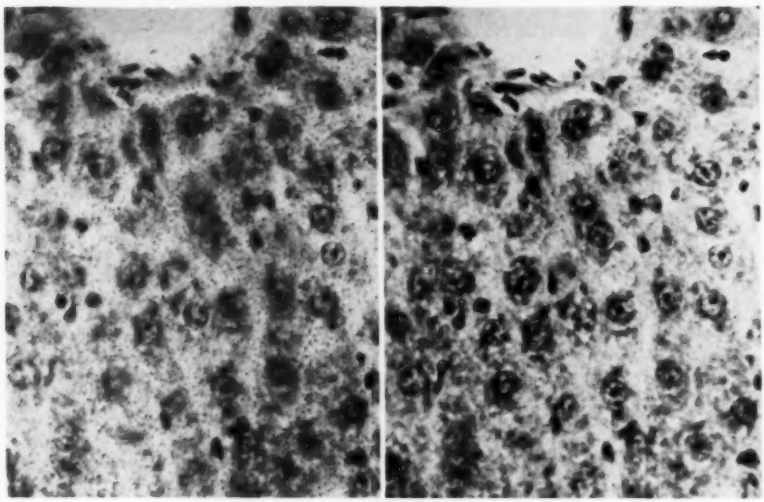
Fig. 1.—Autoradiogram of liver 2 minutes after injection, demonstrating diffuse, lobular pattern of the radioisotope. (Magnification ×20; reduced ½.)

RESULTS

Autoradiograms.—The autoradiograms of the livers from the animals killed at 2 minutes show that the dye had already spread through the entire hepatic parenchyma. There is a moderate variation in concentration, with areas of highest activity located around the portal triads and lesser amounts in the regions of the central veins (Fig. 1). A detailed study showed no focal localization in any particular cell type.

Within 10 minutes the lobular pattern disappeared and at all intervals after this, almost all the radioactivity was diffusely and uniformly distributed throughout the liver parenchyma (Fig. 2). At the 7-minute interval and at all later intervals infrequent spots of concentrated radioactive material appeared in addition to the diffuse pattern. These foci were widely scattered and

appeared to account for a very small fraction of the total radioisotope. They have no consistent distribution in relation to liver architecture. In some instances, determination of the cell type associated with the clumped radioactive particles was not possible; the density of the image in the emulsion obscured the histology. In other examples it could be seen that the foci were more commonly associated with polygonal cells, and less frequently with Kupffer cells. It was not possible to obtain satisfactory photographs demonstrating the activity in relation to specific cell types. Fig. 3 simply shows one of the foci.



A. *B.*

Fig. 2.—Autoradiogram of liver 30 minutes after injection, showing diffuse uniform distribution of the radioisotope. This field does not include any of the rare focal concentrations. *A*, Focused on emulsion. *B*, Focused on tissue. (Magnification ×528; reduced ¼.)

The animals studied at the 3-day interval had very little residual radioactivity, but that present still showed the same pattern; most of the radioisotope was diffusely distributed, with occasional focal concentrations.

Radioassay Results.—A preliminary experiment was done to show in a general way the fate of the total amount of radioactivity injected so that later attention could be focused on the major systems involved. In representative animals, tissue assays indicated that no large portion of the injected dye dose was present in the thyroid, skin, kidneys, spleen, or muscle that could not be accounted for by the blood content of these tissues. The total amount of the radioisotope in the liver was about 2,000 times that in the thyroid and about 400 times that in the spleen. This indicates that there was little free iodine and that the reticuloendothelial system was not important as a site of removal of the labeled dye. It was demonstrated that in all animals, regardless of the time of death, the sum of the activity in the liver, gastrointestinal tract, and plasma was 75 to 85 per cent of the estimated administered dose. Therefore, attention was focused on these compartments.

The total amount of radioactivity in the liver and gastrointestinal tract was measured directly; the content of the plasma was calculated by radioassaying an aliquot and multiplying by a factor based upon the estimated percentage of total body weight accounted for by the plasma.[8]

For the purpose of this study the sum of the activity recovered from the liver, gastrointestinal tract, and contents, plus the amount in the total plasma was considered to be "GLP," and this was arbitrarily considered to be 100 per cent. The data are expressed as percentages of GLP and the results are shown in Fig. 4. The data do not cover a long enough time interval to show the eventual decrease in gastrointestinal content expected to occur due to fecal excretion.

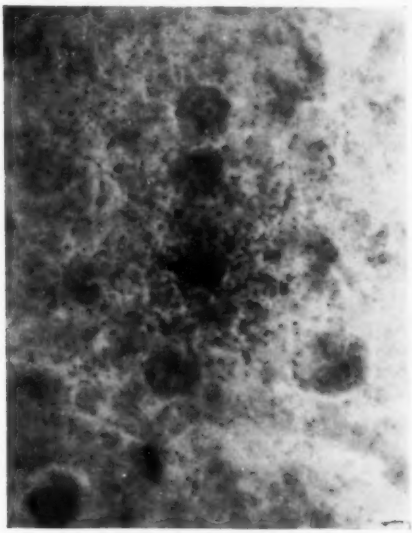


Fig. 3.—Focal concentration of radioisotope in liver parenchyma. Focused on emulsion. (Magnification ×1164; reduced 1/5.)

DISCUSSION

It is seen from these data that as the RBI^{131} finds its way through the circulation it is extracted by the liver at a rapid rate for 15 to 20 minutes at which time the concentration in the liver attains a maximum. After this time the plasma activity remains relatively constant and the liver concentration diminishes as the dye is excreted into the gastrointestinal tract. The build-up in the gastrointestinal tract starts from the earliest time of observation when much of its activity may be due to its blood content and reaches its maximum when its lumen contains most of the material that has been excreted by the liver. The general shape of the liver curve in Fig. 4 approximates Taplin's curves obtained by external counting in rabbits and human beings.[9]

The qualitative information from the autoradiograms supports the accepted concept that RBI^{131} is "cleared" from the plasma as it passes through the liver.

Initially, concentrations of radioactivity are greater near portal triads and the pattern of distribution is somewhat lobular.

The scattered unicellular concentrations of activity seen in the liver 7 minutes after injection of the dye and at all longer intervals are difficult to interpret. It is not clear why certain polygonal cells should contain more activity than others, or why a few Kupffer cells should concentrate the dye.

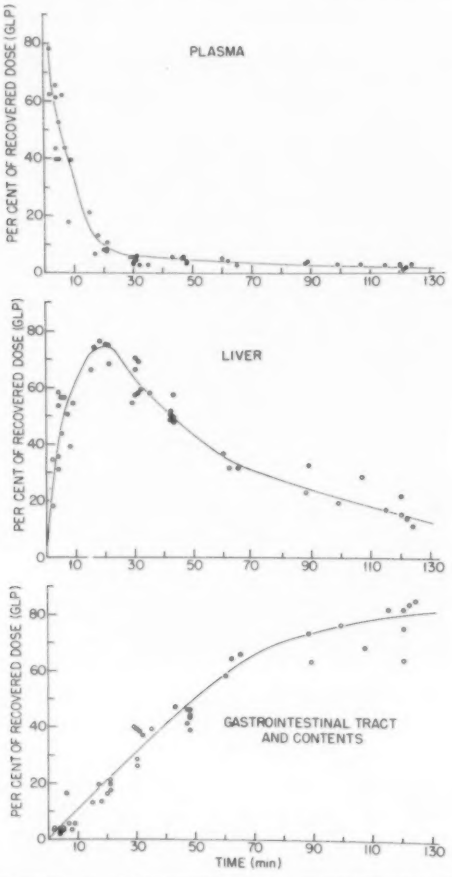


Fig. 4.—Distribution of radioactivity among gastrointestinal tract, liver, and plasma based upon radioassay data. Each circle represents one animal. The curves were fitted visually.

The concentration in Kupffer cells might be the result of engulfment of aggregations of the dye, which might have occurred before injection, or while the dye was circulating in the bloodstream. However, we are unable to suggest any reason for the high concentration seen in some polygonal cells.

The activity remaining in the blood and liver at long intervals has been postulated to be due to complexing of the dye with body proteins.[2]

The results obtained in this experiment were compared with those from an earlier study done in this laboratory (and with the same techniques) on the distribution of a known colloid of Au^{198} given intravenously to rats.[10] As compared with the distribution of colloidal Au^{198} the following features are noted: Au^{198} at very early intervals assumes a lobular pattern of localization with greatest concentration in the region of the portal triads. Although this is grossly similar to that seen with RBI^{131} at those early intervals, microscopically the distribution of the Au^{198} at all intervals is seen to be extremely patchy, and the gold is localized to the reticuloendothelial system exclusively.[11] Furthermore, the lobular pattern of Au^{198} is considerably more pronounced and this pattern is maintained for at least as long as 9 days. This is in contrast to the RBI^{131}, which becomes evenly spread after 7 minutes and is excreted through the biliary system.

SUMMARY

Studies with radioiodine-labeled rose bengal given intravenously to rats showed that the dye was rapidly cleared from the plasma by the liver and excreted into the gastrointestinal tract. The maximum liver concentration was at about 20 minutes. No other tissues showed any important concentration.

Autoradiograms indicated a generally diffuse distribution of the dye in the liver parenchyma. There was no evidence for important reticuloendothelial localization of the material.

We wish to acknowledge the assistance of Joe Gray in performing of radioassays, and of Edgar Cress and John Rafter in assisting with the laboratory procedures.

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STUDIES IN LEUKEMIA

X. Passive Immunization of C3H Mice

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THE accomplishment of inducing leukemia in C3H mice by means of cell-free brain filtrates (1) affords an opportunity for evaluation of immunization procedures to prevent the disease. Experiments on passive immunization of C3H mice with rabbit antisera prepared against the cell-free brain filtrates are herein reported.

MATERIALS AND METHODS

C3Heb mice obtained from the Jackson Memorial Laboratory, C3H mice from Cumberland View Farms, Clinton, Tennessee, and Swiss mice from Harlan Small Animal Farms, Cumberland, Indiana, were used. New Zealand white rabbits were obtained from the Scientific Small Animal Farm, Des Plaines, Illinois.

Antigens.—A 1 per cent suspension of leukemic C3H and Swiss mouse brain in 0.9 per cent NaCl solution (buffered at pH 7.4) was passed through a Seitz sterilizing pad* whose integrity was tested with *E. coli.* For controls, nonleukemic mouse brain, identically prepared, and buffered 0.9 per cent NaCl solution were used. Preparations were also made from a pool of 5 brains of patients who died of acute leukemia and a pool of 5 brains of patients who died of other causes (2 stillbirths, 1 of diabetic acidosis, and 2 of heart failures). Equal parts of Freund's adjuvant (Bacto-Adjuvant, Incomplete, Difco) were added to each of these preparations.

Antisera.—Each antigen was injected into 3 rabbits subcutaneously at 2 sites, 2.5 ml. of antigen being given at 5-day intervals for a total of 3 doses. Ten days after last injection the rabbits were bled. The sera were pooled and used immediately to minimize the loss of labile factors.

Each of the pooled antisera was used to immunize 40 mice by intraperitoneal injection of 0.5 ml. the day before, day of, and day after challenge. Twenty of these mice were then challenged by intraperitoneal injection of 0.5 ml. of a cell-free brain filtrate (CFBF) prepared from a leukemic animal. The remaining 20 were challenged by intraperitoneal inoculation of 0.5 ml. of a tumor cell suspension (approximately 10^6 cells/ml.). The experiment employing antisera to isologous antigen in C3H mice was done in duplicate, and the experiment was repeated to test the effectiveness of antisera against heterologous antigens. Methods of preparation of cell-free brain filtrates and tumor cell suspensions are described elsewhere.[1] Because evidence was adduced that in vitro neutralization of the active CFBF occurs, mice immunized with antisera used in the first replicate were challenged by the intracerebral route.

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*Obtained from A. Daigger & Company, Sheet No. S, pore size range 0.5 μ.

Absorption studies were carried out by incubating 15 ml. of the immune rabbit sera prepared against leukemic C3H cell-free brain filtrate with 1.5 Gm. of homogenized leukemic and nonleukemic brain for one hour at 37° C. Each of the absorbed sera was then used to treat 25 mice the day before, day of, and day after challenge with leukemic C3H cell-free brain filtrate.

RESULTS

Experiments in which C3H mice immunized with antisera prepared against isologous antigen were challenged with CFBF and tumor cells are summarized in Table I. Animals were considered positive for leukemia on the basis of their

TABLE I. C3H MICE DEVELOPING LEUKEMIA

CHALLENGED WITH	TREATED WITH SERUM FROM RABBITS IMMUNIZED		
	WITH LEUKEMIC C3H BRAIN FILTRATE	WITH NONLEUKEMIC C3H BRAIN FILTRATE	WITH SALINE SOLUTION
Cell-free brain filtrate			
Experiment 1	0*/20	14/20	20/20
Experiment 2	0/20	19/20	19/20
Total	0/40	33/40	39/40
Tumor cell suspension			
Experiment 1	12/20	18/20	20/20
Experiment 2	11/20	19/20	20/20
Total	23/40	37/40	40/40

*Number developing leukemia.

classical gross findings.[1] These were repeatedly checked histologically. The results are unchanged after a minimum of 7 months of observations at the time of writing. At present, the observation is continuing.

Serum from rabbits immunized with CFBF of leukemic mice provides significant protection of C3H mice against induction of leukemia by cell-free brain filtrates (Table I). This effect is not due to immunization by mouse brain protein, because serum from rabbits immunized with nonleukemic mouse CFBF does not afford significant protection.* Significant, although less complete protection is afforded mice challenged with tumor-cell suspensions. Here it is also evident that the protection is not simply due to antibodies against mouse brain protein, because serum from rabbits immunized with nonleukemic mouse brain is not significantly more effective than serum from rabbits injected with saline solution. Comparable results have been obtained in a single experiment using antisera prepared against cell-free brain filtrates from leukemic and nonleukemic Swiss mice to immunize Swiss mice (Table II). Experiments comparing the effectiveness of antisera prepared against antigens derived from isologous, homologous, and heterologous sources are summarized in Table III. It is apparent that significant protection is afforded C3H mice by antisera prepared against leukemic Swiss mouse and leukemic human CFBF. Although these antisera are not as effective as those prepared against isologous antigens, it is evident that their effect is not simply due to the brain tissue used, for no significant protection is afforded animals treated with antisera prepared against nonleukemic Swiss mouse and nonleukemic human all-free brain filtrates.

*The effect is not due to neutralization of the agent by the antiserum within the peritoneal cavity, since immunized mice challenged by the *intracerebral* route were equally well protected.

TABLE II. SWISS MICE DEVELOPING LEUKEMIA

CHALLENGED WITH	TREATED WITH SERUM FROM RABBITS IMMUNIZED WITH	
	LEUKEMIC BRAIN FILTRATE	NONLEUKEMIC BRAIN FILTRATE
Brain filtrate	3*/20	19/20
Tumor cell suspension	12/20	20/20

*Number developing leukemia.

TABLE III. EFFECTIVENESS OF ANTISERA PREPARED AGAINST HETEROLOGOUS TISSUE IN PASSIVE IMMUNIZATION OF C3H MICE

TREATED WITH SERUM FROM RABBITS IMMUNIZED WITH BRAIN FILTRATE	CHALLENGED WITH	
	LEUKEMIC BRAIN FILTRATE	TUMOR CELL SUSPENSION
Nonleukemic C3H	17*/20	20/20
Leukemic C3H	4/20	8/20
Nonleukemic Swiss	19/20	20/20
Leukemic C3H	12/20	15/20
Nonleukemic Human	19/20	19/20
Leukemic C3H	11/20	14/20

*Number developing leukemia.

Additional evidence of the specificity of *leukemic* brain as an antigen is found in absorption experiments. When 25 mice were treated with sera from rabbits immunized with C3H leukemic CFBF and absorbed with nonleukemic brain homogenate, 4 developed leukemia. When 25 mice were treated with the same immune sera absorbed with leukemic brain homogenate, 21 developed leukemia.

DISCUSSION

Our experiments did not cast light on the mechanism of protection. Immune serum conceivably contains antibodies to: (1) brain cells or leukemic cells, (2) antigens common to leukemic tissue but absent from nonleukemic tissue,[2] and (3) virus present in cell-free brain filtrate and tumor tissue. The reduced effectiveness of antiserum against challenge by tumor cells could be due to differences in quantity, quality, or accessibility of the virus, or to "autonomy" of the tumor cells. Our pathogenetic studies on the rapid progression of CFBF-induced leukemia indicate transformation of host cells rather than metastasis.[3] The present experiments suggest that antiserum inactivates the transforming agent during its extracellular periods.

The effectiveness of antisera against Swiss mouse and human leukemic CFBF in protecting C3H mice justifies the presumption that the effective antigens are probably similar and represents an additional similarity between human and murine leukemia.

SUMMARY

1. Antiserum was prepared in rabbits against cell-free brain filtrates of leukemic C3H and Swiss mice and human leukemic brain.

2. It affords a high degree of protection of isologous strains against induction of leukemia by cell-free brain filtrates (CFBF) prepared from leukemic brains, and partial protection against leukemia induced by tumor cell suspensions.

3. Antiserum prepared in rabbits against cell-free brain filtrates of non-leukemic mouse and human brain failed to give significant protection to mice challenged with either leukemogenic CFBF or leukemic tumor-cell suspensions.

4. Antisera against Swiss and human leukemic cell-free brain filtrates are less effective than antisera against C3H leukemic CFBF in protecting C3H mice against induction of leukemia by either cell-free brain filtrate or tumor cell suspensions.

5. The data further justify the thesis that human and murine leukemia are similar diseases.

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FAILURE OF VASOPRESSIN TO PRODUCE NORMAL URINE CONCENTRATION IN PATIENTS WITH DIABETES INSIPIDUS

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DIABETES insipidus is considered to be due to insufficient circulating antidiuretic hormone (ADH) resulting from partial or complete destruction of the neurohypophysis. The primary defect consists of the inability of the kidney to excrete hypertonic urine except perhaps under conditions of extreme water deprivation. The implication exists that replacement therapy with ADH (or its equivalent) will restore urine concentration to normal. There is ample evidence in the experimental and clinical literature that such is not the case. Vasopressin usually decreases the output of urine to normal; however, the urine remains relatively dilute.

This report further demonstrates the inability of patients with diabetes insipidus (DI) to produce hypertonic urine within the normal range.

METHODS AND MATERIALS

Experimental.—Seven young men with normal renal function served as controls. Without regard to meals each was given 1,600 to 1,700 ml. of water to drink within 15 minutes. At the end of 75 minutes all the urine passed spontaneously was pooled and the subject was given vasopressin.† The patient voided at 10-minute intervals for 3 periods. Occasionally no urine was passed during the second or third period, but all normal subjects voided at least once during the 30 minutes.

Two adult patients with diabetes insipidus were also subjected to the same procedure. Both had Hand-Schüller-Christian disease and pulmonary tuberculosis, and were receiving antituberculous chemotherapy at the time of the study. Daily urinary output, which ranged from 5 to 8 liters per day in both patients in the untreated state, was reduced to 2 to 3 liters per day by using either snuff 3 to 4 times per day (A. O.), or 0.5 ml. vasopressin-in-oil on alternate days (E. H.). Pituitary snuff was withheld from Patient A. O. 12 hours prior to the study, and vasopressin-in-oil was withheld from patient E. H. for 36 hours prior to the study. These two patients were studied on several occasions with variations in dose and type of posterior pituitary preparation. E. H. was unable to pass any urine for 40 minutes after 120 mμ of vasopressin had been given.

Data shown in Table I for 3 additional patients with DI were taken from hospital records. The water loading procedure for L. J., an adult, was the same as has been described above for the others; but for L. F. and D. J., 2 children, the hydrating procedure varied

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†Pitressin and Pituitrin were furnished by A. McGinty, Parke, Davis and Company, Detroit, Mich.

somewhat. Vasopressin was administered while each was experiencing brisk diuresis. For these last 3 patients only the maximum urine specific gravity, as recorded in the chart, is tabulated.

Urine osmolarity was determined cryographically using the Fiske osmometer.

Clinical.—Records were reviewed of all patients discharged with a diagnosis of diabetes insipidus within the years 1948 to 1959 from both the Veterans Administration Hospital (7 cases) and the University of Minnesota Hospitals (19 cases). Where available (16 out of 26 cases), pertinent data bearing on the effect of vasopressin on urine concentration was abstracted and tabulated along with the clinical diagnoses in Table II. Information on urine concentration after water deprivation was available in 7 of the 16 cases.

TABLE I. EFFECT OF VASOPRESSIN ON URINE CONCENTRATION DURING WATER LOADING

NUMBER	MAXIMUM URINE CONCENTRATION MOSM./L. OR SPECIFIC GRAVITY	VASOPRESSIN DOSE
Normal subjects		
7	617 ± 35 (474-840)	120 mμ I.V.*
Diabetes insipidus		
A.O. (1)	119	120 mμ I.V.
(2)	160	1.5 ml. I.M.†
E.H. (1) ‡	186	120 mμ I.V.
(2)	214	40 mμ I.V.
(3)	300	1.0 ml. I.M.
L.J.	1.010	20,000 mμ I.M.
D.J. (1) §	1.010	500 mμ I.V.
L.F. (1) §	1.007	4,000 mμ I.M.
(2)	1.010	4,000 mμ I.M.

*Mean ± S.D.

†Surgical Pituitrin, 20 μ/ml. (Parke-Davis).

‡Represents 70 min. sample.

§Initial flow was 3.0 and 3.2 ml./min. in Patients D. J. and L. F., respectively, prior to vasopressin injection.

RESULTS

It is clear from Table I that, under the influence of the experimental conditions described, vasopressin produced a hypertonic urine in every normal subject. The mean urine osmolarity (617 ± 35 mOsm./L.) was more than twice that of normal plasma (285 to 300 mOsm./L.). In contrast, in only one study was a patient with DI able to excrete urine isotonic with or slightly hypertonic to plasma (patient E. H.—Study 3). In the other 4 studies urine osmolarity was considerably hypotonic to plasma. Specific gravity measurements were essentially similar. Even Pituitrin, a posterior pituitary extract containing a mixture of oxytocin and vasopressin and, therefore, more closely resembling the composition of neurohypophyseal excretion than vasopressin alone, did not produce urine of normal concentration. Urine specific gravity measurements recorded in the charts of patients with DI who were eating a regular diet and being maintained on vasopressin indicate that these individuals excrete dilute urine most of the time, even when their volume is within the normal range. It is important to point out that each specific gravity recorded in Table II is the highest found for each subject, but that the majority of values fall far below this figure and seldom exceed 1.010. The specific gravity observed after varying

periods of water deprivation likewise indicates poor concentrating ability but it does not seem to bear any relationship to the severity of the condition as measured by the daily urine output in the untreated state.

TABLE II. URINE CONCENTRATION IN PATIENTS WITH DIABETES INSIPIDUS

NAME	SEX	AGE	URINE OUTPUT LITER/DAY		MAXIMUM SP. GR. ON VASOPRESSIN	WATER DEPRIVATION		PRIMARY DIAGNOSIS	ASSOCIATED DIAGNOSES
			AVE.	RANGE	MAINTENANCE	HR.	MAX. SP. GR.		
R. A.	F	56	7.2	4.9-8.5	1.018			?Skull fracture	
C. N.	F	48	6.6	4.9-8.0	1.007			Developed DI 1 yr. postpartum	Essential hypertension, syphilitic aortitis, diabetes mellitus
V. B.	F	40	8.3	7-10	1.020				
L. J.	M	34	10.2	10-12.5	1.015	8	1.002	Idiopathic	Congenital deformity of fourth and fifth lumbar vertebrae
E. H.	M	32	7.2	5.2-9.0	1.030			Hand-Schüller-Christian disease	Pulmonary tuberculosis
A. O.	M	27	7.2	4.5-8.0	1.023			Hand-Schüller-Christian disease	Pulmonary tuberculosis
R. K.	M	12	8.5	6-11.7	1.017			Unknown	
M. R.	F	11	8.9	9-11	1.010	6	1.015	Post encephalitis	
P. H.	F	10	2.7	1.4-4.9	1.013	21	1.008	Teratoma	
M. H.	F	9	4.3	2.2-5.7	1.005	24	1.006	Optic nerve biopsy—"tumor or inflammatory"	
D. F.	M	8	1.0	0.6-1.5	1.013			Unknown	Left hemiparesis, macrogenitosomia praecox
D. J.	F	7	3.8	2.9-5.4	1.015	8	1.003	Unknown	
J. N.	M	6	2.5	1.4-2.9	1.018	6	1.005	Acute megakaryocytic leukemia, Hand-Schüller-Christian disease	
D. B.	M	6	5.0	2.8-7.2	1.002				
L. F.	M	3	4.4	3.8-5.0	1.010	8	1.003	Unknown	
J. S.	M	½	2.0	1.6-2.7	1.010			Unknown	Atrial septal defect, mental retardation

DISCUSSION

This clinical study was undertaken because it had been noted that vasopressin, even in large doses, failed to restore urine concentration to normal in

rats with DI even though urine output was considerably reduced.[1] While not specifically emphasized as such in the reports of other investigators, similar data dealing with diabetes insipidus in human subjects can be found. Thus, Rapaport and co-workers[2] showed that hydropenia in normal individuals resulted in urine concentrations of at least 1,000 mOsm./L., while urine concentrations failed to exceed 777 mOsm./L.[3] in 3 subjects with DI who were maintained on vasopressin and similarly dehydrated and, in addition, were given vasopressin intensively. DeWardener and Del Greco[4] administered vasopressin intravenously to 4 patients with DI using continuous infusion with doses ranging up to 13 mμ per hour. The maximum urine concentration observed in each patient was 352, 347, 374, and 405 mOsm./L., respectively. This is less than the concentration exhibited by normal individuals. The osmolarity values observed in our report, as well as the others studied, do not actually represent the most concentrated urine that these individuals are capable of excreting because, under the experimental conditions described, admixture with a certain amount of dilute urine occupying dead space is unavoidable. However, it can be assumed that this dilution factor is comparable for all patients.

The reason that ADH (or vasopressin) fails to restore urine concentration to normal in subjects with DI is not clear. These individuals (as well as the rats previously studied) tend to excrete urine of decreased hydrogen ion concentration with pH in the range of 6 to 7 (unpublished observations). Since this occurs even when urine volume is controlled by vasopressin it cannot be explained by simple dilution alone. Excretion of urine of decreased acidity and osmolar concentration which cannot be increased appreciably by vasopressin is also known to occur in primary aldosteronism[5] and potassium depletion.[6-8] There is no evidence, however, that controlled diabetes insipidus is associated with potassium depletion. Indeed, there is evidence that rats with chronic DI are in sodium and potassium balance and that the concentration of these electrolytes in muscle is normal.[9] Furthermore, impaired concentrating ability in response to vasopressin has been observed in animals within a few days after neurohypophysectomy.

The question is raised whether polyuria per se can impair the ability of the kidney to concentrate urine. DeWardener and Herxheimer[10] studied the concentrating ability of two normal human male subjects before and immediately after an 11-day period of polyuria, during which urine output averaged 10 liters per day. After the period of polyuria, maximum urine osmolarity decreased 47 to 58 per cent after vasopressin, and decreased 38 to 45 per cent after water deprivation. However, despite this decrease, osmolarity values still ranged between 407 to 512 mOsm./L. These values are considerably higher than those found in the subjects with DI of this report whose urine output until just prior to the study was only 2 to 3 liters per day.

Except during water deprivation, glomerular filtration is normal in most of the diabetic subjects and, therefore, would not be considered a contributing factor. The recent demonstration by Kleeman and associates,[11] that patients with diabetes insipidus can produce hypertonic urine in the range of 573 to 763 mOsm./L. by reducing glomerular filtration below 10 ml. per minute, merely indicates that such a concentrating mechanism, presumably

operating independently of ADH, does exist. However, this drastic reduction in glomerular filtration, which to a lesser degree can be observed in animals with DI subjected to prolonged water deprivation, is so far removed from ordinary circumstances that this concentrating mechanism can hardly be considered in relation to normal physiologic conditions.

Berliner and co-workers[12] believe that ADH prevents the excretion of a hypotonic urine by facilitating the reabsorption of water in the distal tubule. Hypotonic urine entering the distal tubule then becomes isotonic, and finally hypertonic, after passage through the collecting tubule where ADH also facilitates water reabsorption. However, it is in the distal tubule, where the urine changes from the hypotonic to the isotonic state, that the most work is done and the great reduction in flow occurs. Since in these patients with DI vasopressin reduced flow to normal, this particular function of ADH would seem to have been fulfilled. According to this concept of ADH action, the defect would appear to be in the failure of the collecting tubule to respond to vasopressin. Finally, it is conceivable that ADH does not constitute complete hormonal replacement in DI and that one or more factors are still lacking.

SUMMARY

Seven adult males with normal renal function and two patients with diabetes insipidus (DI) were given vasopressin during water diuresis. In every instance control subjects excreted hypertonic urine (617 ± 35 mOsm./L.), whereas the DI patients excreted hypotonic urine ranging from 119 to 214 mOsm./L. in 4 out of 5 studies, with a maximum of 300 mOsm./L. in one instance. Specific gravity measurements on urine of 16 patients with DI maintained on vasopressin for long periods also provide evidence that only occasionally do patients with this disease excrete urine with specific gravity above 1.010 even though the daily urine output is reduced to a normal level. The reason for this failure of vasopressin to produce hypertonic urine within the expected normal range is not known, but it suggests either that the concentrating mechanism of the DI kidney is still defective or that perhaps a factor(s) in addition to ADH is required for restoration of normal tubular concentrating capacity.

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PRELIMINARY STUDIES ON QUANTITATIVE ZONE ELECTROPHORESIS IN STARCH GEL

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THE technique of zone electrophoresis using potato starch gel as the supporting medium was introduced by Smithies in 1955.[1] This method provided a higher resolution of the serum protein fractions than is obtained with other methods of electrophoresis. However, considerable care and skill are necessary to obtain reproducible results. The present work reports a method for the standardization of the buffer and soluble starch used in making the gel, a controlled procedure for staining, and the utilization of reflection densitometry for quantitative estimation of each protein fraction. As a result, with only minor changes in technique, reproducibility can be more easily attained and greater resolution in the separation of fractions can be achieved. In addition, the results of fractionation of certain proteins by starch gel have been compared with those obtained by boundary electrophoresis, other forms of zone electrophoresis, and sedimentation in an analytic ultracentrifuge. A study of normal human sera and preliminary observations of alteration of serum proteins in certain diseases have been carried out.

MATERIALS AND METHODS

Preparation of Soluble Starch.—Since there were major differences in physical properties of the various potato starches which were available commercially, it was necessary to work with one batch in order to achieve standardization. The starch used in these investigations was derived from Idaho potatoes,† since with this material a larger number of protein fractions were resolved than with any other variety tested. This starch contained 0.03 per cent nitrogen and 10.3 per cent water‡ under standard conditions (vide infra).

The requirements given here may not be exactly suitable for other batches of potato starch. Underhydrolysis with this starch yields a gel which is too viscous upon pouring;

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†Batch #584, Magic Valley Processing Co., Twin Falls, Idaho, obtained from Morningstar-Nicol, Inc., 620 W. 51st St., New York, N. Y. A standardized soluble starch is now available from Connaught Medical Research Laboratories, Toronto, Canada.

‡Water content was determined with a C. W. Brabender Rapid Moisture Tester at 103° C. This instrument was lent by the C. W. Brabender Instruments, Inc., 50 E. Wesley St., Hackensack, N. J.

conversely, overtreatment results in a gel which pours easily but lacks strength and is difficult to handle when removed from the molds.

The preparation of soluble starch was carried out in the manner described by Smithies.[1] Materials and containers to be used were preheated to 36.4° C. by placing them in an incubator room, and then 300 Gm. of potato starch was suspended in 600 ml. of acetone and hydrochloric acid (100:1). After 70 minutes, 150 ml. of molar sodium acetate was added with rapid stirring, resuspending the settled starch. The suspension was then filtered on a large Büchner funnel, and the starch was washed with 3 liters of distilled water. The starch was then suspended in 500 ml. of distilled water, allowed to stand 18 hours at room temperature, filtered, washed again with distilled water, then again with acetone, and dried at 70° C. for 4 hours. Other batches of starch may require longer or shorter periods of treatment with acetone-HCl.

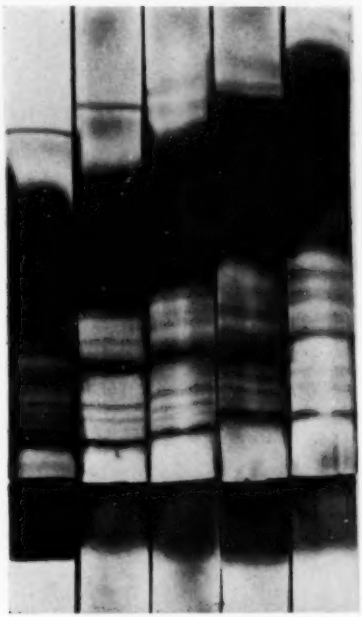

Fig. 1.—The effect of duration of acetone-hydrochloric acid treatment on the electrophoretic pattern obtained from a single normal human serum. From left to right, periods of treatment of 50, 65, 70, 75, and 90 minutes, respectively.

Any temperature between 30° and 40° C. may be selected for the acetone-HCl treatment. Once the temperature has been selected, however, it should not vary more than 0.2° C. for succeeding preparations. Under the conditions used routinely for preparing soluble starch, a change in the temperature of 0.2° C. was found to be approximately equivalent to a 5-minute change in hydrolysis time. The effect upon the electrophoretic separations of such changes in the method of preparation of soluble starch is illustrated in Fig. 1. A temperature low enough to permit a hydrolysis time in excess of one hour should be selected so that the procedure can be timed accurately. Also, since the time the starch stands in water overnight, approximately 18 hours, similarly affects the physical properties of the gel, we found this period should be kept constant within one-half hour.

Gel resulting from a soluble starch produced by the 70-minute acetone-HCl treatment, appeared to give the greatest number of serum fractions with relatively well-defined boundaries.

Preparation of the Starch Gel.—Gels were prepared from soluble starch according to the procedure described by Smithies.[1] In our standard runs 100 ml. of 0.027 M borate buffer, pH 9.05, was added to 13.5 Gm. of soluble starch in a 500 ml. Erlenmeyer flask. The slurry of starch and buffer was heated over a Fisher burner with continuous vigorous agitation until the granules were ruptured and a clear solution was obtained. Bubbles were removed by suction through a one-hole rubber stopper inserted into the neck of the flask and connected through a suitable trap to a water aspirator. This technique requires some experience in order to obtain a clear gel without permitting excessive evaporation and must be carried out in the same way each time. The resulting viscous, translucent fluid was then poured into plastic molds, covered with a sheet of polyethylene, and allowed to cool and solidify. The molds 235 by 36 by 6.5 mm., as described by Smithies,[1] were found to be satisfactory.

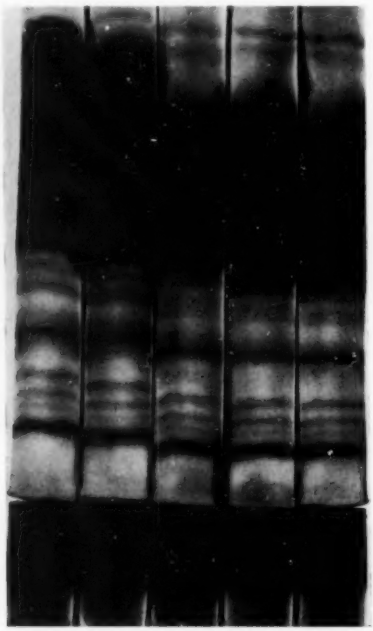

Fig. 2.—The effect of the proportion of starch employed in gels on the electrophoretic pattern obtained from a single normal human serum. From left to right, 12.9, 13.1, 13.3, 13.5, and 13.7 per cent starch.

This gel buffer was prepared by dissolving 1.670 Gm. boric acid crystals (ACS), 10 ml. of N NaOH, and 21 ml. of 0.1 N NaOH in about 700 ml. of distilled water and diluting to one liter. During these studies it was convenient to make gel buffer in 20 L. calibrated flasks. However, if runs using varying buffer conditions are desired, it is more convenient to make up 1 L. of 0.500 M borate with the desired volume of standardized 1 N NaOH added to provide a stock solution. These solutions must be measured accurately, since it has been found consistently that a change of 0.001 M boric acid or a difference of 0.01 pH unit caused definite alteration of the electrophoretic patterns. It should be emphasized, however, that this is not at all difficult to do using ordinary laboratory equipment.

It is well known that the water content of starch varies depending upon conditions of temperature and humidity during storage. During these studies it was observed that the weight of a given sample of soluble starch open to the atmosphere might vary by as much as 6 per cent (0.8 Gm.) on different days with ordinary changes in atmospheric conditions.

A standard sample kept in a covered weighing dish under the same conditions as the samples used for making gels has been found useful in compensating for this variable. When the standard sample weighed 13.5 Gm. its water content was found to be 10.3 per cent. Changes in the patterns resulted if the proportion of starch varied beyond 0.1 Gm. when using 13.5 Gm. in 100 ml. of buffer (Fig. 2).

It is evident that the variations in properties of the gel which depend upon the starch and buffer conditions employed will make a comparison of serum electrophoresis studies among different laboratories difficult. We have, therefore, presented in Fig. 3 the viscosity curve during gelatinization obtained by testing our standard soluble starch and borate buffer with a Brabender Viscograph.* The proportions of the buffer and starch were the same as those used in routine preparation of gels, and the volume of the mixture was 450 ml. which is standard for the instrument. The instrument is used widely in industry for comparing the gelatinization properties of starch[2, 3] and appears to give an accurate method for measurements of the characteristics of gels to be used for electrophoretic studies. These studies will be presented in a subsequent publication.

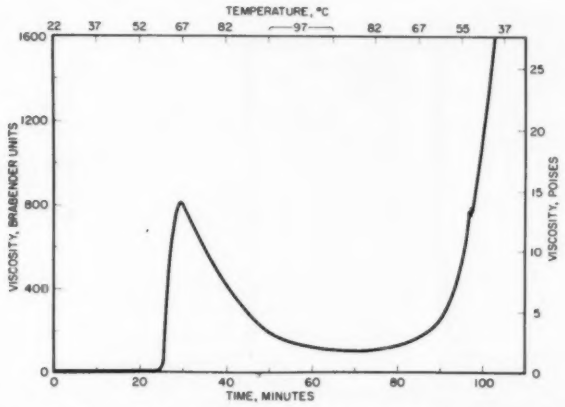

Fig. 3.—Viscosity curve during gelatinization. This curve may be used to characterize the soluble starch and buffer used for gel electrophoresis (see text).

Preparation of a Gel Strip for Electrophoresis.—Four to six hours after preparing the gels, samples were applied by removing a 1.0 to 2.0 mm. wide section transversely at 6 cm. from the cathodic end of the gel. This was accomplished with two razor blades spaced the desired distance apart and held parallel to each other in a surgical needle clamp. The specimen to be tested was mixed with sufficient starch granules to achieve maximum viscosity consistent with easy pipetting into the prepared slit. Considerable care is necessary during application to assure even packing of the starch suspension to minimize *electrodecantation* as the protein enters the gel. The exact volume applied, however, is not critical. The gel was then covered with a thin layer of mineral oil and a piece of 50 gauge industrial Saran film 20 cm. wide was applied, allowing the ends of the gel to be exposed. Electrophoresis was conducted by placing the gels between two jars containing buffer as previously described.[1] Electrical contact was made through the use of thick flannel wicks which extended from the buffer to cover the exposed ends of the gels. The Saran film, being of uniform width, assures that the voltage will be applied across a 20 cm. distance. Flannel wicks were also used to connect the two jars to the electrode vessels containing borate buffer and the platinum electrodes.

*We are indebted to Mr. Blake and Mr. Schmitz of C. W. Brabender Instruments, Inc., Hackensack, N. J., for the use of this instrument.

Conditions for Electrophoresis.—Electrophoresis was carried out at a fixed temperature between 10° and 14° C., usually 12° C., for 16 hours at a constant voltage of 4.5 volts per centimeter. Variation of ± 1° C. resulted in minor changes in the protein fractionation pattern. When electrophoresis was carried out below 4° C. migration was markedly impaired. Runs longer than 8 hours at temperatures over 15° C. resulted in heating which disturbed the pattern in gels of this size. To help obtain a high degree of reproducibility, a specialized power supply was designed for the desired voltage range so that changes in conductivity would not alter the applied voltage. For work not demanding as high a degree of reproducibility, a Heathkit D.C. power supply may be constructed and used.*

Staining Procedure.—The strips were stained for 5 minutes in a saturated solution of napthalene black B 200† in methanol-water-acetic acid solvent (volume 5:5:1) and washed for 4 hours with the solvent. By means of a centrifugal pump the solvent was continuously recirculated through a drum of activated charcoal. Fig. 4 illustrates the apparatus used in this procedure. The advantage of using a solvent which is continuously decolorized is that the procedure is readily standardized and solvent is conserved. A staining time of 5 minutes and 4 hours of decolorizing time consistently yielded maximum contrast in stain-

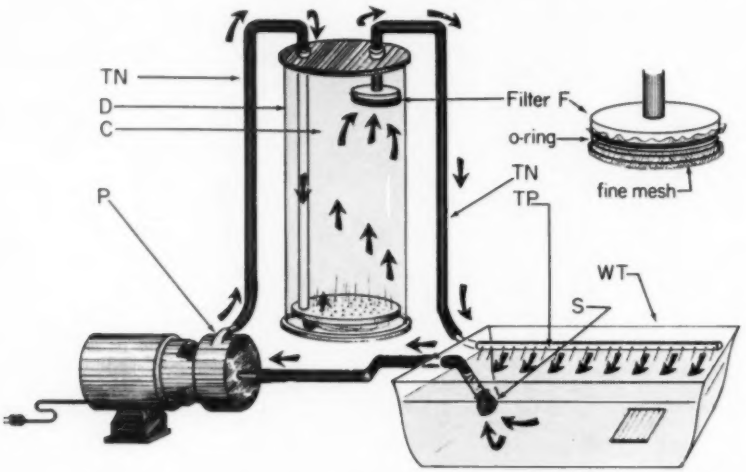

Fig. 4.—Washing apparatus for removing excess stain from gel strips. The solvent containing stain is removed from the bottom of the washing tank through an L-shaped section of plastic tubing protected with a piece of wide mesh screening. This tubing is connected to a centrifugal pump with Tygon tubing. The pump forces the solvent into the bottom of the drum containing activated charcoal. As the solvent passes through the charcoal, it is decolorized and passes out through the filter. It is pumped into the inlet tubing of the washing tank. This piece of plastc tubing is occluded at the end, and the solvent is forced through multiple holes in the tubing to produce a gentle stream of colorless solvent over the gel strips. Since the pressure gradient over the filter is approximately 20 feet of water, resulting in a flow of 4 gallons per minute decreasing to 1 gallon per minute as the charcoal packs around the fiter, it is usually necessary to reduce the flow at the washing tank inlet with a screw clamp. This has been found to be a convenient method for controlling the staining process and for conserving solvent.

Key to abbreviations: WT, washing tank constructed from large plastic refrigeator box and acrylic tubing. TP, Plastic tubing (acrylic), I.D. ⅜ in., O.D. ½ in. TN, Tygon tubing, I.D. ½ in., O.D. ⅝ in. C, Coconut charcoal activated 50 to 200 mesh, Fisher Scientific Company, New York, N. Y. D, Decolorizing drum constructed of acrylic plastic, 5 in. O.D. tubing and ¼ in sheet. Charcoal needs replacement after 6 to 12 months of continuous use. F, Filter constructed of acrylic plastic to provide a filter area 2½ in. in diameter; an o-ring holds the screening in place. Woven Saran fabric No. 342 was generously provided for this purpose by Mr. Robert A. Black, Lumite Division, Chicopee Mills, Inc., New York, N. Y. P, Centrifugal pump and motor, Eastern pump Model B-1, Eastern Industries, Newton, Mass. Polyethylene centrifugal pump No. 5790, Cole-Parmer, Chicago 10, Ill., may be used but requires a filter 4½ in. in diameter of Saran cloth No. 342, backed by a wide gauge stainless steel mesh for support. S, Stainless steel screening.

*Model PS-3, Heath Co., Benton Harbor, Mich.

†Obtained from Arnold Hoffman & Co., Inc., 55 Canal St., Providence, R. I.

ing between the protein fractions and the gel background. Fig. 5 illustrates this point, as verified by reflection densitometry. The stained strips may be stored in Saran wrap for future quantitation.

Reflection Densitometry.—A reflection type densitometer with recorder was developed to make permanent records which could then be used for quantitation of the protein fractions (Fig. 6).[4] The freshly stained gel strip was placed upon a revolving belt which moved at

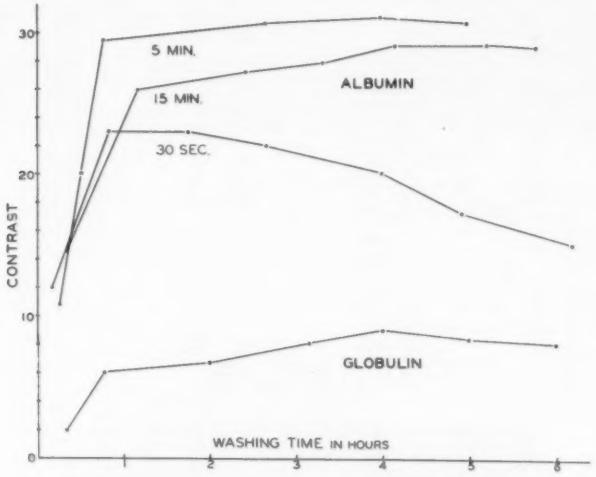

Fig. 5.—Gel staining procedure. Maximum contrast in color between the stained protein fractions and the gel background as measured by reflection densitometry was determined periodically during the continuous washing procedure. Three staining times are illustrated.

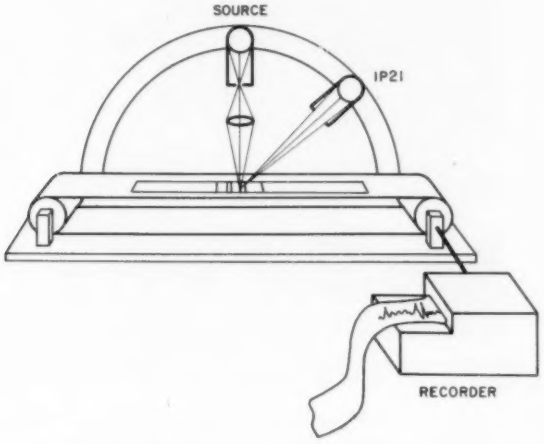

Fig. 6.—Schematic diagram of reflection densitometer. The stained gel strip placed on the revolving belt passes under the light source. The reflected light is detected with the photomultiplier tube (IP21), amplified, and a continuous record of the reflectance is made with a rectilinear recording milliammeter. This instrument was developed in collaboration with the Department of Biophysics of the Airborne Instrument Laboratories, Mineola, Long Island, N. Y.

a rate of 6.5 cm. per minute. An unfiltered white light source was used, and a beam of light 1 by 4 mm. was focused upon the surface of the gel. The reflected light was detected with a photomultiplier tube, 1P21, placed at a suitable angle. Thise optical equipment was mounted within a lightproof box. The signal from the photomultiplier tube was amplified and then

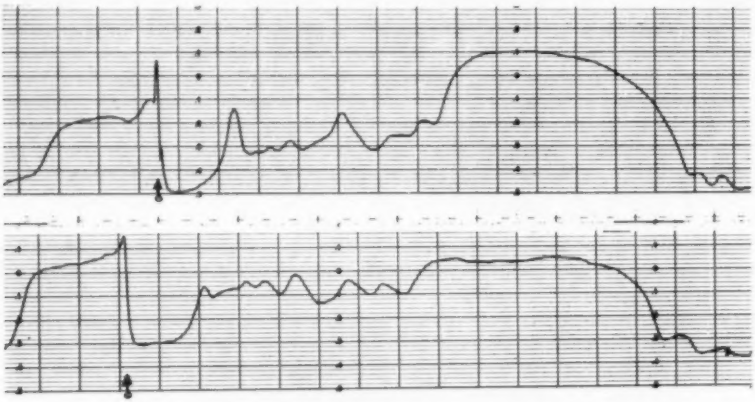

Fig. 7.—Reflection densitometry records of serum proteins. Reflectometer records illustrating the differences in serum protein between normal (haptoglobin type IIB), above, and metastatic carcinoma of the ovary of the same serum type, below.

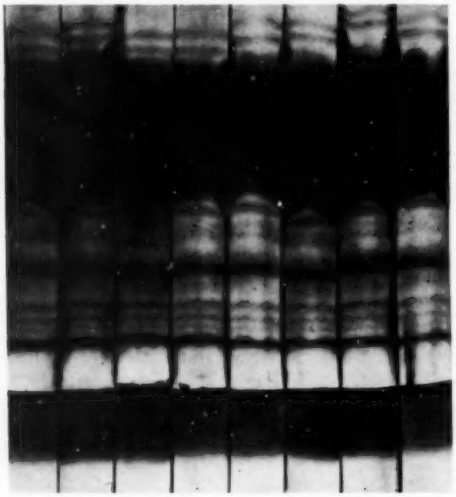

Fig. 8.—Repeated runs with the same serum showing the degree of reproducibility obtained with standardized conditions.

recorded graphically with a rectilinear recording milliammeter.* With the light source perpendicular to the gel strip, light scattering from imperfections in the surface of the gel was minimized, and the photomultiplier tube could be placed at any point over a wide angle

*Texas Instrument, Inc., 6000 Lemmon Ave., Dallas, Texas.

without significant loss of sensitivity. Fig. 7 illustrates two recordings made with this reflectometer and demonstrates the type of records obtained in a normal individual and in a patient with a disease which altered the serum protein pattern from normal.

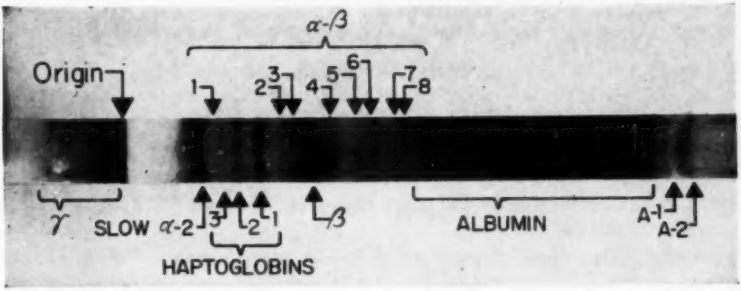

Fig. 9.—Detailed picture of the normal (IIB) serum separated by zone electrophoresis in starch gel to illustrate the nomenclature used.

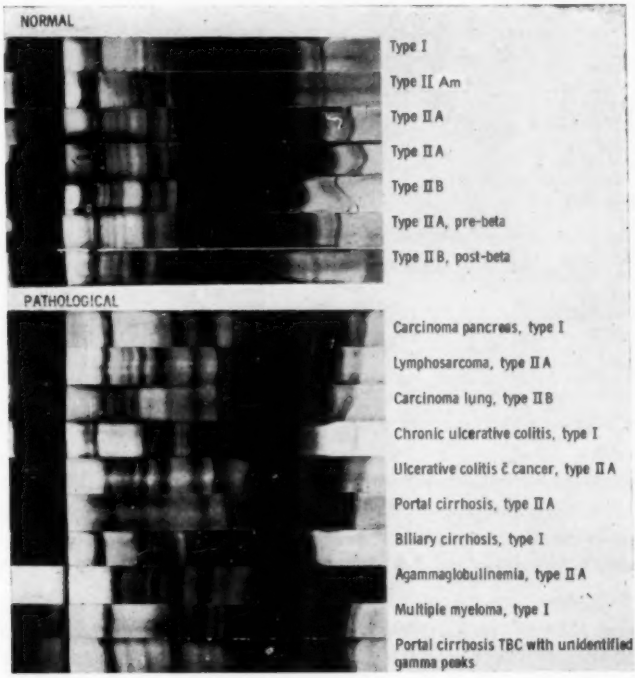

Fig. 10.—Illustration of normal variations and the types of change seen in disease.

RESULTS AND DISCUSSION

Nomenclature, Reproducibility, and Measurement of Fractions.—Using the particular batch of hydrolyzed starch employed routinely through the studies,

a control serum was analyzed in gels prepared under standard conditions but on different days (Fig. 8). Ten of the patterns thus obtained were analyzed with the reflectometer, and the area under the curve for each fraction was determined by planimetry. The percentage of the total reflectance of each fraction was measured, and the mean for each of the 10 strips was calculated (Table I). The standard deviation was calculated by the formula S.D. $= \sqrt{\frac{d^2}{N-1}}$. The two prealbumins, albumin, beta globulin, the haptoglobins, slow alpha-2 globulin, and gamma globulin have been clearly defined by Smithies[1] and were readily found in our patterns. The location of the fast alpha-2 globulin and the post-albumin components was not certain in our gels. For purposes of discussion we have applied the numbering system illustrated in Fig. 9 to the fractions which usually appeared in our patterns of normal sera. These designations should remain flexible until such time as the physiologic significance of the fractions is elucidated.

TABLE I. REPRODUCIBILITY OF THE TECHNIQUE. MEASUREMENTS OF NORMAL SERUM PROTEIN FRACTIONS EXPRESSED AS PER CENT OF THE TOTAL REFLECTANCE

FRACTION	MEAN (PER CENT)	STANDARD DEVIATION
Gamma	19.40	2.08
Slow alpha-2	2.27	0.63
Alpha beta-1	0.22	0.10
Haptoglobin 3	0.69	0.25
Haptoglobin 2	0.99	0.32
Haptoglobin 1	1.51	0.23
Alpha beta-2	1.02	0.25
Alpha beta-3	1.00	0.20
Beta	3.87	0.44
Alpha beta-4	1.89	0.34
Alpha beta-5	1.59	0.37
Alpha beta-6	1.22	0.18
Alpha beta-7	2.16	0.31
Alpha beta-8	0.71	0.28
Albumin	60.15	1.58
A-1	0.64	0.17
A-2	0.64	0.49

Ten electrophoretic runs on the same serum, type IIB, were carried out and the per cent of the total protein for 17 fractions was estimated by planimetry of the reflectance curves. Additional fractions which migrated within the gamma globulin and albumin were included as part of these proteins.

Although the fractions measured do not constitute all of those which can be visually identified, and the measurements do not constitute a quantitative analysis of the proteins, these measurements do serve to illustrate that many low concentration fractions can be identified and that the patterns can be reproduced.

Normal Human Serum Types.—Over 100 normal sera have been analyzed. The 3 genetically determined haptoglobin types previously described,[5] haptoglobins I, IIA, and IIB, were observed. In addition to the haptoglobin types, other normal serum protein types were encountered (see Fig. 10). One such infrequent genetic type encountered in our laboratory and described recently by Smithies[6] was readily identified because of the presence of a "post-beta" globulin component. Another type was the rare "pre-beta" globulin type.[7] Neither

of these was related to the haptoglobin types. In about 80 per cent of Negroes, an additional fraction was encountered between the beta globulin and albumin. This change was associated with a very low concentration of type IIA haptoglobin, such that it might be misinterpreted as haptoglobin type I, and we have tentatively designated it as IIAm. The proteins involved in such serum types were of the order of 3 per cent of the total protein as approximated by reflectance measurement. Thus, they may be readily identified. There were, however, many low concentration fractions which were quite reproducible in the same individuals studied repeatedly over 3 years but which varied quantitatively and qualitatively among different individuals. Such variation is illustrated in the two examples of normal type IIA serum in Fig. 10.

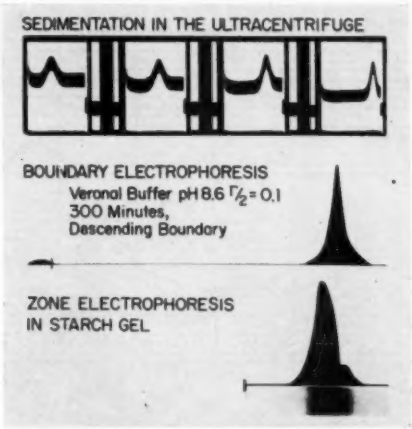

Fig. 11.—Comparison of methods of protein analysis. A 2 per cent solution of Bence-Jones protein sedimented as a single component in the Spinco analytic ultracentrifuge, 0.2 M NaCl buffered at pH 7.4 with 0.01 M phosphate. By moving boundary electrophoresis at 6 pH values, no definite demonstration of heterogeneity was encountered above pH 3. The run with veronal buffer $\Gamma/2$ 0.1 at pH 8.6 is shown above. With the starch gel technique, two distinct components are noted using the electrophoretic conditions standard for serum studies.

Serum Patterns in Disease.—Over 400 sera from patients with a variety of diseases have been studied, and triplicate runs of 100 of these sera gave consistent patterns. Definite variations from normal serum patterns were observed in sera from these patients. Several examples of these pathologic sera are shown in Fig. 10. Often changes are observed in fractions of low concentration making it difficult to distinguish these changes from normal variations. It will be necessary to identify and measure these fractions of low concentration before slight deviations from normal can be characterized.

Resolution and Artifact.—The high resolving power of the method was recognized by Smithies when he found that over 14 serum proteins could be identified. Because of the large number of fractions separated, it has been suggested that many of the bands might be artifacts. In order to get further information on these points, several proteins, including Bence-Jones protein,

serum fractions isolated from starch block, and purified enzymes were investigated using the ultracentrifuge, boundary electrophoresis, and filter paper electrophoresis. The results were compared with those obtained by starch gel eletrophoresis. All 30 Bence-Jones proteins tested were heterogeneous by the starch gel method. In analyzing one of these, we were unable to demonstrate heterogeneity by sedimentation (Spinco ultracentrifuge, 0.2 M NaCl buffered at pH 7.4 with 0.01 M phosphate) or by boundary electrophoresis at pH 8.6, 7.6, 6.0 (ionic strength 0.1 buffers),[8] although more than one component was observed below pH 3. However, by starch gel electrophoresis using conditions standard for the serum studies described above, two components were noted (Fig. 11).

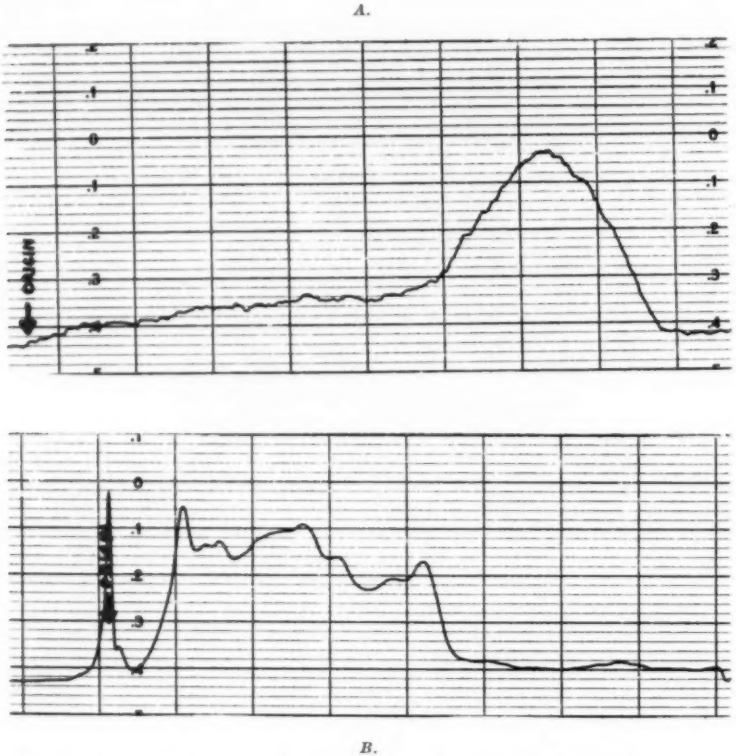

Fig. 12.—A comparison of filter paper (*A*) and starch gel electrophoresis (*B*). Alpha-2 globulin, isolated by starch block electrophoresis, appearing as a single component with paper electrophoresis (*A*) but showing at least 8 fractions with starch gel (*B*).

A serum protein isolated from the center of the alpha-2 globulin peak of a starch granule electrophoresis block appeared as a single fraction on standard paper electrophoresis* but showed at least 8 fractions on starch gel (Fig. 12).

*Spinco Model R, paper electrophoresis system, Spinco Div., Beckman Instruments, Inc., Belmont, Calif.

However, when a highly purified enzyme, ribonuclease-A, similar to the specimen prepared by C. H. W. Hirs by column chromatography[5] was subjected to starch gel electrophoresis, it appeared as a single fraction* (Fig. 13). As with many fractions migrating toward the cathode, slight trailing was noted. It is a relatively stable protein so that similar studies will have to be made with serum fractions to be certain that none of the small components appearing in starch gel electrophoresis are breakdown products of serum proteins.

Adaptation of the Procedure.—If it is desirable to test for the presence of heterologous proteins at concentrations of 1 per cent or less of the major component, conditions for electrophoresis may be altered, since accurate comparability is not necessary. The slit for sample application may be widened to 1 cm. to allow for larger sample volume, a somewhat "underhydrolyzed" starch may be used to produce sharper peaks, and the duration of the run may be selected to permit the particular protein under study to migrate a centimeter or two but not so far that peaks become flattened. At present, this method can be used to good advantage in testing for protein homogeneity. The supporting medium, the buffer, and the electrophoretic conditions can be altered with little difficulty. The method does not seem to be useful, however, for

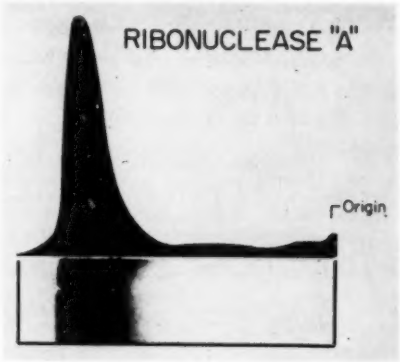

Fig. 13.—This highly purified enzyme migrated as a single fraction in starch gel electrophoresis. Trailing is noted with many proteins migrating toward the cathode.

determining mobilities or isoelectric points. In addition, some proteins, such as ribonuclease and insulin, were difficult to stain with present methods, diffused rapidly, or were soluble in methanol. Other proteins, such as fibrinogen, some macroglobulins, and some gastric mucoproteins, remained at the origin and did not migrate into the gel, probably because of their molecular size or shape.

SUMMARY

1. An analysis of the problems encountered in obtaining reproducible electrophoretic separation of proteins in starch gel has shown that there are critical limits for the preparation of soluble starch, buffer, and proportions of these

*We are indebted to Dr. Stanford Moore of the Rockefeller Institute for Medical Research for making this sample available.

used in making the gels. These limits are easily obtained with ordinary laboratory equipment, and an awareness of those steps in which accuracy is needed will permit considerable modification without loss of reproducibility.

2. Conditions of electrophoresis which employ temperatures lower than room temperature but above freezing and an 18-hour application of voltage resulted in the separation of more protein fractions than were described previously in normal serum.

3. Semiautomatic staining procedures using continuously decolorized solvent for elimination of excess dye provide reproducible staining of starch gel electrophoretograms.

4. An analysis of the reflectance curves obtained for normal serum electrophoretograms by use of a specially designed reflection densitometer demonstrated that a high degree of reproducibility can be obtained.

5. A survey of 100 normal and 400 pathologic sera indicates that while there are many normal serum types, each with complex patterns, the changes in disease are sufficiently marked that this method holds considerable promise for further studies. Because of the marked variation in normal serum protein patterns, accurate information regarding the changes specific to a given disease will require a quantitative method of gel strip analysis and adequate data processing procedures.

6. When employed in testing the homogeneity of several purified proteins, zone electrophoresis in starch gel gave rapid and sensitive results comparable with or better than those obtained by other methods.

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STUDIES ON THE HETEROZYGOUS CARRIER IN GALACTOSEMIA

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GALACTOSEMIA is a hereditary disorder of carbohydrate metabolism characterized by vomiting, diarrhea, jaundice, poor weight gain, and malnutrition during early infancy. Mental retardation, cataracts, and cirrhosis of the liver are sometimes seen in this condition. Clinically, the diagnosis is usually established on the basis of an abnormal galactose tolerance curve and the presence of galactose in the urine. In 1956, Schwarz, and associates[1] showed that galactose-1-phosphate (Gal-1-P) accumulates in the erythrocytes of children with galactosemia, if they receive galactose or milk. In the same year, Isselbacher and associates[2] demonstrated a deficiency of Gal-1-P uridyl transferase in the erythrocyte of these children. This indicated that the site of the metabolic block in this disease was at the step of:

Galactose-1-phosphate + UDP glucose $\rightleftharpoons$ UDP galactose + Glucose-1-phosphate.

The frequent occurrence of this condition in siblings and among the offspring of consanguineous matings together with its equal distribution in both sexes suggest that galactosemia is probably transmitted by a single autosomal recessive gene.[3] In this situation, the heterozygous carriers for the abnormal gene will usually be free of clinical symptoms. Careful biochemical studies, however, might reveal minor departures from the norm.[4] Holzel and Komrower[5] undertook galactose tolerance tests among parents, siblings, and close relatives of 6 families with many cases of galactosemia. They found the response to be abnormal in a number of the relatives tested, but this was not a consistent finding among all the individuals who should be heterozygotes. Furthermore, in the majority of families, only one of the parents showed an elevation of the galactose tolerance test. Their more recent studies have suggested that the determination of hexose phosphate after a galactose load might prove to be useful as a diagnostic tool.[6] In a preliminary communication,[7] we have shown that the heterozygous carriers for galactosemia can sometimes be detected by a deficiency of Gal-1-P uridyl transferase activity in the erythrocytes.

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*Fellow, Schweppes Foundation, Chicago, Ill.

The purpose of the present paper is to compare the relative merits of galactose tolerance tests, the measurement of hexose phosphate levels after such a load, and the direct determination of blood Gal-1-P uridyl transferase activity as means of detecting the heterozygous carriers in galactosemia.

METHODS

Determination of Blood Galactose.[8, 9]—Whole blood (0.2 ml.) was hemolyzed with 2.8 ml. of distilled water. Eighty units of glucose oxidase was added to the hemolyzate and the mixture was incubated for 30 minutes at 37° C. At the end of the incubation period, protein was removed by the addition of 0.4 ml. of 0.3 N NaOH and followed by 0.4 ml. of 5 per cent $ZnSO_4$. Two milliliters of filtrate was placed in a tube calibrated to 25 ml. followed by 2 ml. of alkaline copper reagent. The solution was then heated in a boiling water bath for 10 minutes. After cooling, 1 ml. Nelson's arsenomolybdate reagent was added and the solution was diluted to 25 ml. with distilled water. Colorimetric determination was made at a wave length of 515 mμ in an Evelyn photoelectric colorimeter. For each determination a series of galactose standards were run simultaneously.

Isolation and Chromatography of Sugar Phosphate Esters.[10, 12]—Blood was hemolyzed as in the assay of Gal-1-P uridyl transferase activity. Two milliliters of hemolyzate was deproteinized with an equal volume of 10 per cent trichloroacetic acid. The filtrate was adjusted to pH 8.2 by the addition of 1/300 volume of 10 per cent phenolphthalein and addition of 1 N KOH to adjust to a discernible pink. Five-tenths milliliter of 25 per cent barium acetate was added to the filtrate and the suspension was chilled for 15 minutes and centrifuged. The precipitated material was not homogenous and contained in addition to the barium-insoluble compounds, some of the barium-soluble materials in the form of double salts. These can be removed by redissolving the precipitate, and then reprecipitating it as follows: the crude barium-insoluble precipitate was dissolved in 0.1 N HCl. A drop of barium acetate solution was added and the pH adjusted to 8.2 with KOH. The suspension was chilled again for 15 minutes and centrifuged. The supernatant contained the barium-soluble materials, which had been precipitated with the barium-insoluble compounds, and was added to the original barium-soluble fraction. This barium-soluble fraction (including the soluble materials from the reprecipitation) was treated (pH 8.2) with 4 volumes of 95 per cent ethyl alcohol, chilled for one half hour, and the precipitate removed by centrifuging. The barium salts were redissolved in 0.4 ml. of 0.1 N HCl, the Ba^{++} was removed as $BaSO_4$ and the solutions were neutralized to litmus with NH_4OH. Portions equivalent to 0.2 to 0.3 ml. of blood were placed on Whatman No. 1 paper. The chromatogram was run in n-propanol-ammonia-water (60:30:10 v/v) for 17 to 18 hours, air dried, and then rapidly drawn through acid molybdate reagent in acetone. It was heated for 3 minutes in an oven at 85° C., placed under an ultraviolet lamp for 10 minutes, and finally exposed to water vapor, when the blue spots due to hydrolyzed phosphate appear. The amounts of glucose phosphate and galactose phosphate were determined on a Spinco Analytrol scanner and compared with known amounts of galactose-1-phosphate run simultaneously. The hexose phosphate values represent the sum of galactose-1-phosphate and glucose-1-phosphate in the erythrocytes.

Assay of Gal-1-P Uridyl Transferase Activity.[13]—This method is essentially that of Anderson and co-workers.[13] Venous blood (about 5.0 ml.) was drawn into a heavy duty Pyrex centrifuge tube containing 0.15 ml. of heparin. The sample was kept cold in an ice bath, and then centrifuged in the cold at 600 g for 15 minutes. Plasma was aspirated off and the red blood cells were washed twice with a double volume of cold 0.9 per cent NaCl and then hemolyzed by the addition of an equal volume of cold distilled water. Hemolysis was completed by freezing and thawing. Twenty-five hundredths milliliter of hemolyzate was then incubated with galactose-1-phosphate (0.375 μM), uridine diphosphoglucose (0.14 μM), and tris buffer pH 8.1 for 30 minutes at 37° C. For each sample, a control was run

in which no galactose-1-phosphate had been added to the substrate. At the termination of the incubation period 1 ml. of distilled water was rapidly added, and the mixture was heated at 100° C. for 2 minutes with rapid and constant stirring with a glass rod. The sample was then cooled and centrifuged. The amount of uridine diphosphoglucose remaining in the heat-inactivated supernatants was determined by means of uridine diphosphoglucose dehydrogenase in the presence of diphosphopyridine nucleotide (DPN). The increase of optical density at 340 mμ, brought about by the reduction of DPN to DPNH, was measured until the optical density reached a maximum, which usually takes about 20 to 30 minutes. The difference between the changes of O.D. at 340 mμ of the blank and the sample constitutes the basis for the calculations of Gal-1-P uridyl transferase activity.

One unit was defined as that amount of enzyme which would convert one micromole of UDPG (uridine diphosphoglucose) to UDP galactose per hour. Units of Gal-1-P uridyl transferase per milliliter of hemolyzate are obtained as follows:

$$\frac{(\Delta\ 1' - \Delta\ 2') \times V \times t}{f \times v \times E}$$

$\Delta\ 1'$ = difference between initial and final reading at 340 mμ (control)*
$\Delta\ 2'$ = difference between initial and final reading at 340 mμ (sample)*
$V = 1.6$ ml. (total volume of incubation mixture at end of incubation)
$t = 2$ (factor to bring incubation period to one hour)
$f = 12$ (by the method used, 1 μm of UDPG per milliliter converted would correspond to a Δ O.D.$_{340}$ of 12.0)
$v = 0.3$ ml. (volume from incubation mixture used for assay of UDPG)
$E = 0.25$ ml. (volume of hemolyzate used in incubation mixture)

For each hemolyzate, the hemoglobin was determined, and diphosphoglucose pyrophosphorylase was run to assure that the blood sample and the hemolyzate had been properly handled. The Gal-1-P uridyl transferase was expressed as units per gram of hemoglobin.

RESULTS

Table I shows the oral galactose tolerance test in 4 normal controls, 8 heterozygotes, and 6 galactosemic children. These were done by the administration of 40 Gm. of D-galactose in adults and 1.25 Gm. per kilogram body weight

TABLE I. FASTING AND 1- AND 2-HOUR BLOOD GALACTOSE LEVELS (EXPRESSED AS MILLIGRAMS PER CENT) AFTER RECEIVING A GALACTOSE LOAD*

SUBJECT	NORMAL SUBJECTS			HETEROZYGOTES			HOMOZYGOTES		
	F	1 HR.	2 HR.	F	1 HR.	2 HR.	F	1 HR.	2 HR.
1	1	28	1	8	28	6	7	86	64
2	4	26	2	7	31	10	0	266	207
3	4	48	4	8	17	10	9	38	116
4	1	21	2	10	46	10	8	125	164
5				4	53	8	2	138	168
6				5	43	40	9	92	92
7				7	69	6			
8				8	0	0			
-									

*This was done by the administration of 40 Gm. of D-galactose orally in adults and 1.25 Gm. per kilogram of body weight in children.

in children. Venous blood was drawn prior to the load, and one and two hours afterward. The galactose tolerance test was regarded to be normal if the blood galactose values returned to the fasting level within two hours. The galactosemic children had a high elevation of blood galactose after the load and this

*Both of the readings are corrected for nonspecific density changes at 2½ minutes and at termination of assay.

elevation of galactose was not lowered by the end of the two hours. The heterozygotes showed a similar response as in the normal person. Only in one case, there was a slight persistence of blood galactose by the end of the two hours after the load.

TABLE II. BLOOD HEXOSE PHOSPHATE LEVELS (EXPRESSED AS MILLIGRAMS PER MILLILITER) OF NORMAL SUBJECTS, HETEROZYGOTES, AND HOMOZYGOTES, PRIOR TO AND ONE AND TWO HOURS AFTER RECEIVING A GALACTOSE LOAD*

	NORMAL SUBJECTS			HETEROZYGOTES			HOMOZYGOTES		
	FASTING LEVEL	INCREMENT OVER FASTING LEVEL		FASTING LEVEL	INCREMENT OVER FASTING LEVEL		FASTING LEVEL	INCREMENT OVER FASTING LEVEL	
SUBJECT	(MG./ML.)	1 HR.	2 HR.	(MG./ML.)	1 HR.	2 HR.	(MG./ML.)	1 HR.	2 HR.
1	6.9	3.1	−1.0	4.6	2.7	0.5	2.3	7.8	11.0
2	5.2	3.4	1.8	3.2	2.2	−0.5	1.1	9.1	7.2
3	3.2	1.1	0.6	5.4	3.6	1.8	1.5	8.8	6.7
4	6.5	4.5	0.5	3.8	0.7	0.5	0.5	7.4	4.9
5	4.2	4.1	2.3	3.3	2.5	0.6	2.7	6.8	8.3
6				3.0	1.5	0.9			
7				6.5	3.3	−0.5			
8				4.2	2.0	1.2			
9				3.7	1.1	−0.5			

*This was done by the administration of 40 Gm. of D-galactose orally in adults and 1.25 Gm. per kilogram of body weight in children.

TABLE III. GAL-1-P URIDYL TRANSFERASE ACTIVITIES (EXPRESSED AS UNITS PER GRAM OF HEMOGLOBIN) IN 10 GALACTOSEMIC FAMILIES

	PARENTS		AFFECTED CHILDREN		UNAFFECTED CHILDREN	
FAMILY	FATHER	MOTHER	1	2	1	2
Families Where Parents Are Heterozygotes						
1	1.8	1.7	0.6	1.0	1.8	2.5
2	3.2	2.6	0.3	0.0	—	—
3	3.8	5.4	0.0	—	3.2	—
4	5.5	4.5	0.0	1.1	—	—
5	2.5	1.9	3.6*	—	—	—
6	2.5	3.8	1.5	—	—	—
7	3.0	1.9	1.0	—	—	—
8	2.2	2.2	0.8	0.0	—	—
9	3.0	2.1	0.0	0.8	—	—
Families Where Offspring Are Heterozygotes						
	PARENTS					
	FATHER	MOTHER			OFFSPRING	
10	0.9	3.4			1.7†	
					2.1	
					4.1	
					2.5	
					1.1	
					3.4	

*Samples taken from infant weighing 3 kilograms after receiving 195 ml. of whole blood.
†Mother of the galactosemic family No. 1.

The hexose phosphate levels in normal controls, heterozygotes, and galactosemic children after receiving the galactose load are given in Table II. A high elevation (three- to fivefold increase above fasting levels) of this fraction of phosphate esters was observed in the galactosemic children after the galactose load. It is reasonable to assume that a large portion of this increment was due to galactose-1-phosphate. In both the heterozygotes and normals, the elevation of hexose phosphates was moderate and returned to normal by the end of 2 hours.

The Gal-1-P uridyl transferase activity for 23 heterozygotes (17 were parents, 5 were children, and one was both a parent and child of patients with galactosemia) and 15 galactosemics are tabulated in Table III and summarized together with those for 16 normal controls in Table IV. The values for this enzyme run higher than those initially reported by Anderson and associates[13] because of the quantity of UDPG used and the method of calculation. The difference between normals and heterozygotes is highly significant ($t = 3.0$; d.f. $= 37$; $P = 0.005$). It is apparent that the difference has become more significant with increasing numbers of subjects as compared with our preliminary communications.[7]

TABLE IV. SUMMARY OF GAL-1-P URIDYL TRANSFERASE ACTIVITIES (EXPRESSED AS UNITS PER GRAM OF HEMOGLOBIN) OF NORMAL SUBJECTS, HETEROZYGOTES, AND HOMOZYGOTES

	NORMAL SUBJECTS	HETEROZYGOTES	HOMOZYGOTES
Number of subjects	16	23	14*
Mean	4.3	2.9	0.6
S.D.	±1.4	±1.1	±0.5
Range	2.8-7.0	1.1-5.5	0.0-1.5

*The affected child with blood transfusion was excluded.

In previous studies, Hsia and associates[14] have shown that the heterozygous carrier for phenylketonuria can be detected by the administration of L-phenylalanine. Because of the possibility that the enzyme activity can actually be altered under conditions of stress, Gal-1-P uridyl transferase activity of venous blood was determined prior to, and one and two hours after, the load. It would appear that galactose had no effect upon the enzyme activity as shown in Table V.

TABLE V. GAL-1-P URIDYL TRANSFERASE ACTIVITIES (EXPRESSED AS UNITS PER GRAM OF HEMOGLOBIN) OF 8 HETEROZYGOTES, PRIOR TO AND ONE AND TWO HOURS AFTER RECEIVING 40 GM. GALACTOSE

SUBJECT	FASTING	ONE HOUR	TWO HOURS
1	1.7	1.6	1.7
2	1.8	1.0	0.9
3	3.2	2.5	1.8
4	2.6	2.9	2.2
5	3.0	3.0	3.2
6	1.9	2.2	2.2
7	2.2	1.5	2.8
8	2.2	1.0	2.7

DISCUSSION

Although clinically galactosemia has always appeared to be transmitted by a recessive mode of inheritance, up to the present time biochemical confirmation has been lacking. Newcomb and associates[3] have undertaken a genetic analysis of all the cases of galactosemia reported in the literature. After excluding 36 families with only one child, the data on the remaining 57 families was analyzed by the a priori or Apert's method. Using Hogben's tables reproduced by Neel and Schull[15] for a recessive mode of inheritance the expected number of affected children was found to be 81.3 with a total variance of 22.3. The number actually observed in these families was 88. The difference between the observed and the

expected, 7, divided by the square root of the variance, 4.72, gave the "t" value which in this case was 1.5. A "t" value of 1.5 with 7 degrees of freedom, there being 8 different family sizes in the analysis, was not significant.

Although there were slightly more men than women reported with this condition, the difference did not reach a significant value, either when compared with the unaffected sibs or the expected number, calculating this from the ratio of a large series giving the sex incidence at birth.

This genetic analysis would seem to suggest that galactosemia is transmitted by an autosomal recessive gene.

In the past, Neel[16] and Hsia[4] have pointed out that the heterozygous carriers of abnormal recessive genes may be detected by minor abnormalities. The present data on Gal-1-P uridyl transferase activity offers evidence that a biochemical difference exists between the heterozygotes and the normal controls. While the results in a single individual must be interpreted with caution, the lower values of Gal-1-P uridyl transferase found in a group of heterozygotes offer confirmation of the view that the lack of this enzyme is the primary biochemical lesion in galactosemia. Furthermore, the decrease of this enzyme in the heterozygote with one abnormal gene and its virtual absence in the homozygote with two abnormal genes strongly suggest that the condition may be transmitted by a single recessive gene.

In the past, Holzel and associates[5] have suggested that the heterozygous carriers for galactosemia might be detected by means of galactose tolerance tests. Their more recent studies[6] indicate that the determination of hexose phosphate levels after a galactose load might also prove to be helpful. The present data indicate that direct measurement of the enzyme activity might prove to be a more sensitive index of abnormality. This fits with the general concept that in an inborn error of metabolism, assay of the enzyme itself is more likely to reveal the abnormality than measuring the compounds immediately preceding the site of the metabolic block. It has the further advantage of being a safer test among galactosemic patients than the galactose tolerance test.

Komrower and associates[6] and Isselbacher[17] have studied the retention and excretion of galactose in patients with galactosemia and found that an appreciable amount of galactose is utilized by such individuals. Furthermore, the time of onset of symptoms and also the degree of severity vary considerably among the patients. This suggests that in galactosemic patients, the ability to convert galactose-1-phosphate to glucose-1-phosphate is not completely lacking. While some of these findings can be in part explained by the accessory pathway, the pyrophosphate reaction shown by Isselbacher,[18] our studies have shown that there is a wide range of Gal-1-P uridyl transferase activity among affected individuals, and the metabolic block may be incomplete. This may in part explain why only some of the heterozygous carriers are detectable by these methods. The possible relationship between the degree of mental retardation and the Gal-1-P uridyl transferase activity in galactosemic children is being reported in a separate paper.[3]

SUMMARY

Three approaches, galactose tolerance tests, hexose phosphate levels after a galactose load, and blood Gal-1-P uridyl transferase activity, have been used to determine whether a biochemical difference exists between the heterozygous carriers in galactosemia and normal controls.

The data show that while the results in a single individual must be interpreted with caution, the values for Gal-1-P uridyl transferase appears to be the most sensitive index for showing an abnormality among heterozygotes. This offers further confirmation that the lack of Gal-1-P uridyl transferase activity is the primary biochemical defect in galactosemia, and that the condition is most probably transmitted by a recessive gene.

We wish to express our appreciation to Grace Lawrence, M.S., for the galactose determinations and to the many physicians who referred their patients to us for this study.

ADDENDUM

Since the completion of the present manuscript, two other papers have appeared describing the detection of the heterozygous carrier in galactosemia. The data in the first report[19] were based upon a slight modification of the assay procedure of Anderson and co-workers.[13] Kirkman and Bynum[20] depended upon a new method of assay for Gal-1-P uridyl transferase based upon oxygen uptake; they used supplementary phosphoglucomutase.

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EFFECT OF PHENYLHYDRAZINE ON THE SIDEROPHILIN AND IRON LEVELS IN SERA OF RABBITS

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SINCE we were interested in the possibility of studying in rabbits the phenomenon of serum iron and bilirubin rise in serum following intravenous administration of nicotinic acid, as occurs in human beings (Laurell[1]), we treated rabbits with acetylphenylhydrazine to establish an initial low serum iron level. While subsequent injection of nicotinic acid into such rabbits failed to elicit the response anticipated, associated hematologic and serum protein studies revealed some interesting effects of phenylhydrazine. We wish to summarize these observations in this report.

MATERIALS AND METHODS

Rabbits of approximately 2 kg. weight received from 8 to 12 mg. of acetylphenylhydrazine subcutaneously per kilogram of body weight for 4 days. During these days and for the subsequent 13 to 15 days, venous blood samples (about 5 ml. each) were taken from the ear and analyzed for hemoglobin, hematocrit, and bilirubin values; reticulocyte, total white cell, and differential counts; serum iron levels, siderophilin concentrations, and electrophoretic serum protein values. Hemoglobin was determined as cyanhemoglobin (Drabkin and Austin[2]); bilirubin, by the method of Jendrassik and Grof[3]; serum iron and siderophilin concentrations by the method of Schade and associates.[4]

RESULTS AND DISCUSSION

Fig. 1 presents the average values for hemoglobin and hematocrit plotted as the per cent of their initial levels and for the reticulocyte counts plotted as per cent of the daily total red cell population of 6 test and 2 control animals. The days on which phenylhydrazine was administered to the test animals are indicated by arrows. The hematocrit and hemoglobin values fall simultaneously and precipitously over the first 5 days to approximately 50 per cent of their starting values but recover rapidly following cessation of drug administration. The hematocrit recovery precedes that of hemoglobin and maintains its advantage throughout the recovery period. Whether this fact reflects solely the effect of the greater volume per cell and increasing population of the reticulocytes over the remaining mature red cells or includes as well an inhibition of the normal rate of bone marrow hemoglobin

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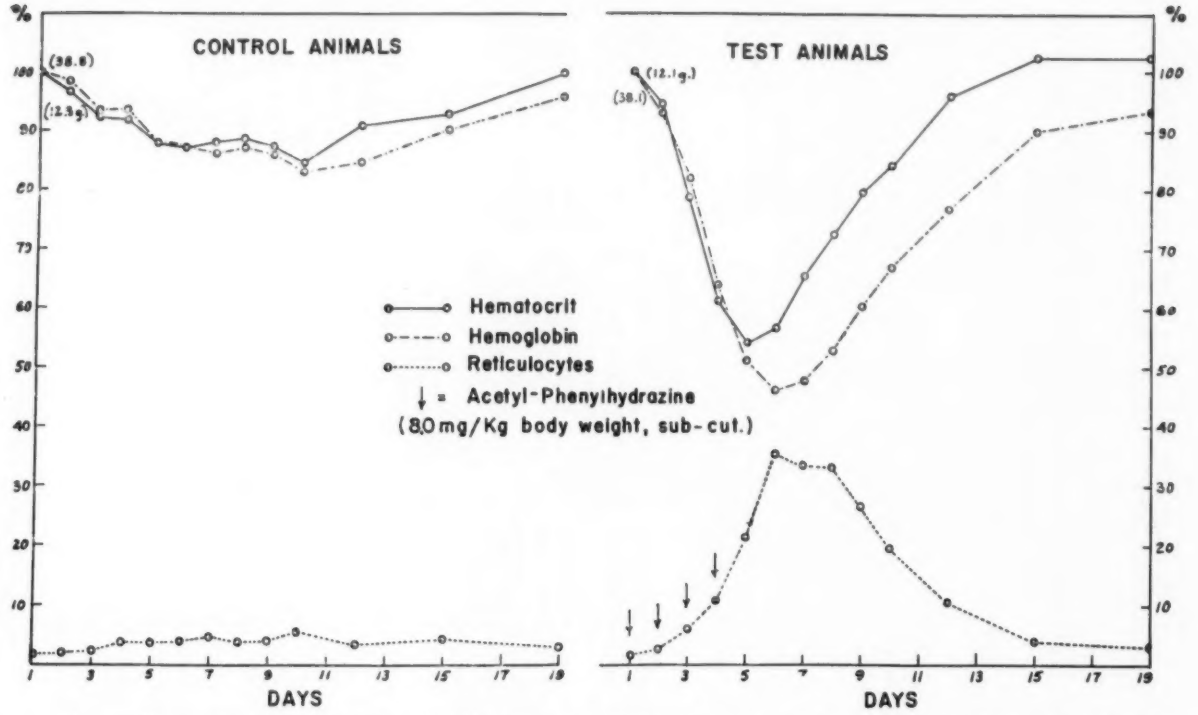

Fig. 1.—Changes in hematocrit and hemoglobin levels and reticulocyte counts as a result of phenylhydrazine injections. The values are averages from 3 control and 6 test animals. Hemoglobin and hematocrit are plotted in terms of per cent of the initial indicated values. The reticulocyte values are percentages of the total daily erythrocyte population.

production by phenylhydrazine during the days of its administration cannot be answered with certainty on the basis of these data. The results do, however, indicate that phenylhydrazine does not significantly interfere with the stimulation of the bone marrow to produce new red blood cells or with their production.

The total white cell count began to rise modestly on the third day of treatment to reach a peak by the sixth day of 166 per cent of the initial level and then to fall to a normal value by the eighth day. The differential white cell count showed no shift in the relative proportion of component cells throughout the full period of observation.

The usually low level of bilirubin in these rabbits (range 44 to 90 μg per cent) was slightly elevated from the fourth through the eighth day with a sharp rise to, and a fall from, a maximum of approximately 300 μg per cent on the fifth day.

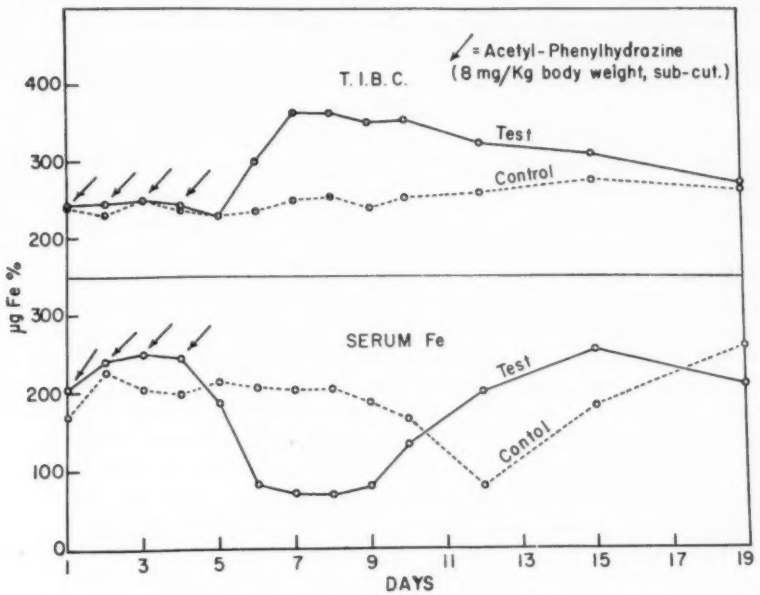

Fig. 2.—Changes in total iron binding capacity (T.I.B.C.) and serum iron levels resulting from phenylhydrazine administration. The changes observed in the control rabbits are the effects of repeated 5 ml. blood samplings.

Investigation of the serum iron levels and siderophilin concentrations as determined by the total iron binding capacity (T.I.B.C.) of the various sera gave some expected as well as unexpected results. The data presented in Fig. 2 were obtained from bled, but untreated, control rabbits as compared with bled and treated test rabbits. Considering first the bound or serum iron curves, it is evident that during the first 4 days of drug administration (Days 1 through 4) the serum iron level was maintained high. In fact, if, as is

usual, the initial value prior to drug administration was below the maximum iron binding capacity of the serum, the serum iron concentration rose to, and slightly above, the T.I.B.C. of the serum under the influence of phenylhydrazine. By the day following the last injection of the drug (the fifth day), the serum iron level had already begun to fall sharply to reach a minimum of only 33 per cent of the normal values by the eighth day. However, the recovery to normal levels was almost equally as rapid. The particular shape of the curve undoubtedly represents in the main a resultant of the rates of iron release from the hemoglobin breakdown by the reticuloendothelial system and the demand for iron by the bone marrow to produce new red blood cells. The fact that the serum iron level remained, during the period of drug treatment, at its maximum height despite the evidence of immediate significant synthetic activity of the bone marrow again suggests that phenylhydrazine may have exerted an inhibitory effect on the synthesis of hemoglobin. The fall in serum iron level in the controls by the twelfth day reflects the bone marrow response to the accumulated blood loss sustained by the rabbits.

Directing our attention now to the T.I.B.C. curves in Fig. 2, we note a remarkable increase in siderophilin concentration in the test animals subsequent to the end of drug administration. The siderophilin began to rise on the sixth day to reach a maximum of 150 per cent of the normal values on the seventh day and remained elevated over a total period of 7 days. The failure to increase in concentration before the sixth day might be interpreted as an indication of phenylhydrazine interference with either the synthesis or mobilization of siderophilin. However, the more or less coincident fall in serum iron with the rise in T.I.B.C. is similar to what is seen in iron deficiency anemia and, more particularly, to what has been reported (Colehour and associates[5]) to occur in rats when subjected to high altitude conditions. In general terms, an acute demand for hemoglobin production leads not only to the reduction of serum iron concentration but also, in nonfasting animals, to an increase in siderophilin concentration in the blood (i.e., elevated T.I.B.C.). The mechanism of the later phenomenon is completely obscure although its consequence for iron metabolism may be of importance for absorption of iron from the intestinal tract, exchange of this metal with the body iron stores, and the availability of serum iron to the bone marrow.

Having observed the increase in concentration of siderophilin in blood serum as evidenced by increase in its total iron binding capacity, we were especially interested in other possible changes in the over-all serum protein composition resulting from phenylhydrazine administration to rabbits. Paper electrophoretic analyses of serum samples revealed no changes in the proportionate amounts of the gamma, beta-2, or alpha proteins in the collected test sera or in any of the protein fractions of the control rabbit sera. However, the albumin and the beta-1 globulin fractions of the test sera, as shown in the curves in Fig. 3, varied significantly from their initial values. For the first 5 days, including 4 days of drug administration, the albumin and beta-1 globulin fractions remained at an essentially constant level of approximately 53 and 8 per cent, respectively. On the sixth day, the concentration of both

proteins shifted so that by the eighth day the albumin level fell to 44 per cent and the beta-1 globulin rose to 12.5 per cent. It should be noted that the approximately 50 per cent rise in the beta-1 fraction as shown by electrophoretic analysis corresponds to the 50 per cent rise in siderophilin concentration as indicated by the T.I.B.C. analysis. On an absolute weight basis, however, only 45 per cent of the beta-1 protein fraction increase is ascribable to siderophilin. We have no information to show that the concomitant fall and rise of albumin and beta-1 globulin fractions, respectively, are causally related but it appears likely that their behavior is part of the complex of reactions associated with greatly accelerated hematopoiesis.

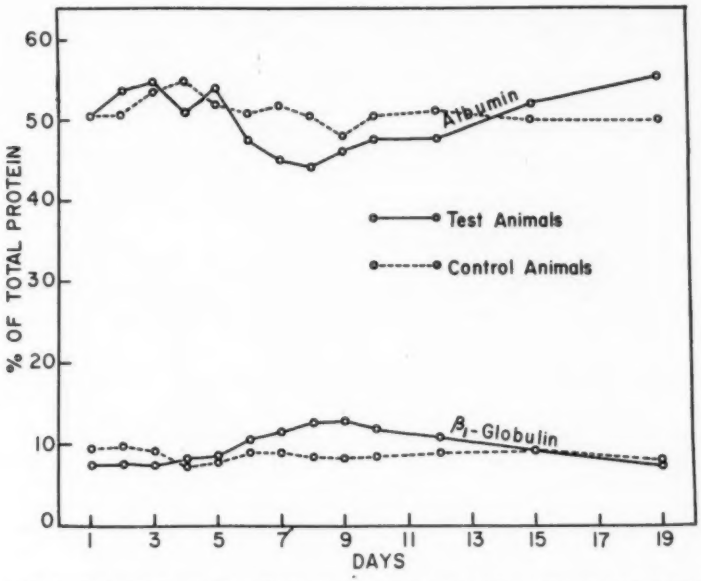

Fig. 3.—The effects of phenylhydrazine injections on serum albumin and beta-1 globulin. The drug was administered on Days 1, 2, 3, and 4. The results, plotted as percentages of total serum protein, were obtained by filter paper electrophoretic analysis.

SUMMARY

1. Blood of phenylhydrazine-treated rabbits was analyzed for (1) hemoglobin, hematocrit, and bilirubin values, (2) reticulocyte, total white cell, and differential counts, and (3) serum iron, siderophilin, and electrophoretic serum protein values during and following drug treatment.

2. Serum iron remained at siderophilin saturation levels during the 4-day period of drug administration and fell to one-third the normal concentration 4 days after cessation of drug treatment. Recovery to normal levels (about 80 per cent saturation) was equally rapid. These variations in serum iron have been considered in relation to temporally associated hemoglobin, hematocrit, and reticulocyte values.

3. Total iron binding capacity of the sera of the test animals, unchanged through the days of drug treatment, rose to 150 per cent of the initial value 3 days following cessation of drug administration. The coincident high total iron binding capacity and low bound iron values are suggestive of the conditions obtaining in iron deficiency anemia and in animals responding to high altitudes.

4. Electrophoretic analyses of the serum proteins from the test animals showed a reduction of about 10 per cent in the concentration of albumin and a rise of about 50 per cent in that of the beta-1 globulin fraction 3 to 4 days following the end of the phenylhydrazine treatment. No changes were observed in the concentrations of the other serum protein fractions.

We wish to thank Mr. John R. Miller for his initiative in this project and his valuable technical assistance as well as that of Mrs. Irene Clark and of Mr. Robert W. Reinhart.

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CONCENTRATION OF PENICILLIN IN ISCHEMIC TISSUE AFTER INTRAMUSCULAR INJECTION

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THE concentration of systemically administered antibiotics in tissues whose arterial circulation is impaired has not been determined to our knowledge. Current methods of study of arterial circulation with radioactive isotopes[1] would not appear to supply satisfactory parameters for the acquisition of such data. Information of this type is basic for the assessment of the value of systemic therapy with antibiotics in patients who have infected ischemic limbs. The need for such data prompted this study.

MATERIALS AND METHODS

Selection of Patients.—A total of 18 adults undergoing amputation of 20 limbs because of arterial insufficiency constituted the source for tissues and blood in which the levels of penicillin were studied. None of the patients had urinary obstruction or evidence of renal disease; none was receiving any other antibiotic or chemotherapeutic agent. Urinalyses were performed routinely and the level of blood urea was determined when indicated to give an additional estimation of renal function. The salient clinical features in these patients are recorded in Table I.

Dose and Administration.—The dose of procaine penicillin G was 600,000 units injected intramuscularly at intervals of 12 hours. Four patients (Cases 1, 4, 8, and 11) received only 2 injections preoperatively, but the remaining patients received 3 or more injections. Blood levels were measured 2 to 6 hours after the last dose of penicillin. Excision of tissue from the distal and proximal ends of the amputated limbs occurred usually within the subsequent 30 to 60 minutes. Since the time relationships between the procurement of blood and of tissue were relatively constant, the temporal discrepancies between the obtaining of the samples and the last dose of the antibiotic given would not appear to be significant. Whenever a delay was to occur between the collection of the material to be studied and the assay, the material was stored in the frozen state in a chamber refrigerated with solid carbon dioxide. Prior to refrigeration, the samples of blood were centrifuged under sterile conditions and the serum was collected.

Method of Assay.—Assays for penicillin were carried out by the cup-plate method, using *Sarcina lutea* as the test organism and penicillin G as the standard. Stock solutions of penicillin G were made up in phosphate buffer at pH 7 and were kept in a chamber refrigerated with solid carbon dioxide. When serum was assayed, all dilutions of the serum to be assayed and of the standard were made in pooled human serum.

The assay of tissue for penicillin and other antibiotics has been described previously by our group.[2-4] In the present study, the freshly obtained tissues were weighed and ground in

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TABLE I. DATA ON 18 PATIENTS UNDERGOING AMPUTATION OF 20 LIMBS BECAUSE OF ARTERIAL INSUFFICIENCY

CASE	AGE (YR.)	SEX	TYPE OF ARTERIAL DISEASE	DIABETES	DEGREE OF ISCHEMIA	MOST DISTAL PALPABLE PULSE	LEVEL OF AMPUTATION
1	77	F	ASO*	Yes	Gangrenous	Femoral	Above knee
2	25	M	TAO†	No	Pregangrenous	Femoral	Below knee
3‡	54	M	ASO	No	Gangrenous	Iliac	Above knee
					Gangrenous	Iliac	Above knee
4	57	M	ASO	Yes	Pregangrenous	Femoral	Above knee
5	63	M	ASO	No	Pregangrenous	Femoral	Trans-metatarsal
6	67	M	ASO	Yes	Pregangrenous	Popliteal	Below knee
7	71	M	Embolic arterial occlusion	No	Gangrenous	Femoral	Above knee
8	40	M	TAO	No	Pregangrenous	Femoral	Below knee
9	75	F	ASO	Yes	Pregangrenous	Popliteal	Above knee
10	54	M	ASO	No	Gangrenous	Femoral	Above knee
11	27	M	Traumatic severance of artery	No	Gangrenous	Femoral	Above knee
12	76	M	ASO	No	Intractable ischemic pain	Femoral	Below knee
13‡	43	M	Embolic arterial occlusion	No	Gangrenous	Femoral	Above knee
					Pregangrenous	Femoral	Above knee
14	56	F	Embolic arterial occlusion	No	Gangrenous	Aortic	Below knee
15	76	M	ASO	No	Pregangrenous	Femoral	Above knee
16	83	M	ASO	No	Pregangrenous	Femoral	Above knee
17	81	M	ASO	No	Pregangrenous	Femoral	Above knee
18	55	M	TAO	No	Pregangrenous	Femoral	Below knee

*ASO = Arteriosclerosis obliterans.
†TAO = Thromboangiitis obliterans.
‡Bilateral amputations.

TABLE II. DIFFUSION OF PENICILLIN INTO TISSUES OF AMPUTATED LIMBS AFTER MULTIPLE* INTRAMUSCULAR INJECTIONS OF 600,000 UNITS EVERY 12 HOURS

CASE	PENICILLIN (UNITS/ML. OF SERUM)	PENICILLIN (UNITS/GM. OF TISSUE)	
		PROXIMAL	DISTAL
1	0.48	0.13	Trace
2	0.56	0.24	Trace
3†	0.48	0.12	0
		0.15	0
4	1.15	0.22	0.21
5	1.25	0.32	0.16
6	1.9	0.2	0
7	Not done	0.29	0.24
8	2.2	0.58	0.1
9	2.3	0.17	0.6
10	2.6	0.38	0.03
11	2.95	0.39	0.25
12	3.0	0.26	0.7
13†	3.1	0.54	0
		0.53	0
14	3.4	0.42	0.94
15	4.4	0.49	0
16	4.7	0.74	1.02
17	4.8	1.88	0.72
18	6.0	1.2	1.4
Mean‡	2.6 ± 0.4	0.46 ± 0.14	0.32 ± 0.12

*Four patients (Cases 1, 4, 8, and 11) received only 2 injections preoperatively; all others received 3 or more.
†Bilateral amputations.
‡The value following the ± sign is the standard error of the mean.

a mortar with a measured amount of pooled normal human serum. These mixtures were stored in the refrigerated chamber. At the time of assay, the samples were thawed and lightly centrifuged. The supernatants were serially diluted in normal human serum, and the various dilutions then were assayed in the manner previously described.

RESULTS

The concentrations of penicillin in serum and in the proximal and distal tissues of the amputated limbs of these patients after they had received multiple doses of the antibiotic are summarized in Table II. Measurable levels of antibiotic activity (mean value of 2.6 units per milliliter) were found in the serums of all the patients. Detectable amounts of penicillin also were found in all the samples of tissues excised from the proximal part of the 20 amputated limbs. Samples of tissues from the distal part of 14 limbs also demonstrated antibiotic activity.

COMMENT

This study, designed to determine if penicillin, when present in the blood, reaches the tissues of limbs with impaired arterial circulation, has provided data apparently worthy of further investigation and consideration. In two thirds of the cases, penicillin was present in the distal tissues of such limbs when detectable amounts of the antibiotics were present in the serum. In the proximal tissues of the limbs in this study, the concentration of penicillin averaged 15 to 20 per cent of the concentration in serum; the distal tissues of the limb usually had a lesser concentration than did the proximal tissues. The concentration of penicillin in the proximal tissues of ischemic limbs when compared to the concentration in serum may not be greatly different from levels of penicillin in tissue and serum in persons with apparently normal arterial circulation.[5]

Occlusive arterial disease may be complicated by lesions that are grossly infected; effective control or eradication of such infection may result in the salvage of a limb. It has been our clinical impression that certain infected ischemic lesions, particularly in diabetic patients, have been benefited by appropriate antibiotic therapy. The results of our study appear to support this clinical impression, in that detectable concentrations of penicillin usually were found in the ischemic tissues. When penicillin-sensitive organisms are present in infected ischemic lesions, administration of this agent would appear to be indicated. The topical application of solutions containing antibiotics cannot be encouraged because of possible harm to ischemic tissues, possible induction of allergic reactions, and lack of penetration into the underlying tissues, in which the infection therefore persists.

We are in the process of determining whether the results obtained with penicillin can be duplicated with other antibiotics, such as the tetracyclines, erythromycin, and novobiocin.

Criticism might be raised that we have measured the amount of antibiotic present in the blood vessels of the specimens examined rather than the concentration of the agent in the tissues themselves. This observation does not appear to be pertinent, in view of the fact that if the penicillin gained entrance into such obstructed arteries, then the compromised tissues, if infected, could have

been susceptible to its antibacterial effects. In other words, the value of antibiotics in patients with infection in ischemic limbs might be denied a priori, because it might be concluded that such deficient circulation could not contain significant amounts of an antibacterial agent. If perhaps we have measured the amount of antibiotic contained in the vessels of the tissues examined, we have demonstrated that compromised arterial circulation usually can continue to bring detectable amounts of an antibacterial agent to infected tissue.

SUMMARY AND CONCLUSIONS

A study of the concentrations of penicillin in the tissues of 20 limbs amputated after parenteral administration of penicillin indicates that tissues whose blood supply is compromised usually contain detectable amounts of systemically administered penicillin. Regardless of whether the tissues themselves contain the penicillin or whether the vessels in the tissues contain the agent that has been measured, administration of penicillin to patients with such limbs who have infections susceptible to this agent can be recommended.

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STANDARDIZATION OF THE PULMONARY NITROGEN CLEARANCE TEST USING OLDER NORMAL MEN

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THE quantitative estimation of impairment of pulmonary function in emphysema is a problem which has received considerable attention in recent years, and has been well summarized by Ebert.[1] The most widely applied tests for disability in emphysema measure restrictions of air flow (maximum breathing capacity and timed vital capacity tests), increased residual volume, and derangement of blood gases. A less widely available test procedure which has considerable promise is the measurement of rate of pulmonary nitrogen clearance during oxygen breathing over a period of several minutes. This has evoked clinical interest because it reveals striking and significant differences between the responses of normal individuals and patients with emphysema. Single breath nitrogen clearance tests have also been devised[2] but are not the subject of the present discussion.

The pulmonary nitrogen clearance test as here described yields measurements of the efficiency of intrapulmonary gas mixing (distribution of inspired gas), as well as of the functional residual capacity and residual volume. The information obtained may be expressed simply as the time or volume needed to dilute alveolar nitrogen to a given end point.[3] Since the size of the lungs, differences in breathing patterns, and especially hyperventilation affect washout times, a more quantitative measurement is the turnover ratio, which is the volume required to dilute the alveolar nitrogen to a given end point divided by the lung volume so washed out.[4-6] By more elaborate analysis the results may yield information on the size and number of well ventilated and poorly ventilated lung compartments and the rates at which they are ventilated.[7-9] A critique of these various methods has been made by Svanberg[10] and Bouhuys and associates.[11]

In a previous study in this laboratory[6] a pulmonary nitrogen clearance test suitable for clinical use was standardized for a group of normal young men and the results compared with other published values. However, since pulmonary emphysema is essentially a disease of advancing age and since it is widely believed that many apparently normal older individuals have

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emphysematous tendencies, it is necessary that standards and distributions for nitrogen washout in normal older persons be studied. The present report reveals our findings in such a group.

MATERIALS AND METHODS

Eighteen vigorous male hospital employees from the professional staff, chiefly physicians, were selected as free from significant pulmonary disease on the basis of a normal chest roentgenogram and the absence of pertinent history and symptoms. Their ages ranged from 37 to 67 years. One 80-year-old patient, free of emphysema, was added to the group. The mean age for the entire group was 51.7 years. The characteristics of the subjects are given in Table I.

The methods used were similar to those previously described.[6] The subjects were studied in the supine position with slight elevation of the head and shoulders. An open-circuit system of gas exchange was used, passing through a four-way respiratory valve with a mouthpiece. Through two passages of this valve the subject first breathed room air, the tidal movements being recorded by a small spirometer attached to a balloon-in-bottle device.[12] At the end of a normal expiration, the valve was switched to the other two passages, allowing the subject to inspire oxygen from a bag and to discharge his expired gas into a 120 liter Tissot spirometer. Simultaneous recording was made of the end-tidal nitrogen concentration of each expired breath (by means of a nitrogen meter with a recording milliammeter*) and the volume of each expiration (by means of a kymograph attached to the Tissot spirometer) during a 7-minute period of oxygen breathing, the procedure being terminated by a final forced expiration at the end of the seventh minute. The peak end-tidal nitrogen was considered as alveolar and designated as $F_{A_{N_2}}$. Simultaneously with the automatic recording of $F_{A_{N_2}}$, end-tidal carbon dioxide was measured automatically at the mouth by an infrared carbon dioxide analyzer (in the first 5 subjects end-tidal air was collected in a syringe for micro-Scholander analysis for carbon dioxide). Spirometer nitrogen and carbon dioxide concentrations were measured in the first 5 subjects chemically as well as by meter; in the remaining 14 subjects all gas analyses were by meter only, accuracy being based on constancy of meter calibration readings with known reference gas mixtures.† The carbon dioxide measurements were used to calculate "anatomic" dead space by the Bohr formula, thereby deriving the fraction of total ventilation which is alveolar (V_A/V_E).

In our method the relatively slow response of our direct writing nitrogen meter recorder necessitates sampling of end-tidal nitrogen at a point beyond an expiratory flap-valve close to the mouth (point "A"). In an effort to determine whether significantly different results would be found if sampling were done directly at the mouthpiece (point "B"), in trials with 10 subjects two nitrogen meters were employed simultaneously, one sampling at point "A" and the other at point "B," both recording on a two channel Sanborn oscillographic recorder. No significant difference could be found, from which it is concluded that for practical purposes the smaller, less expensive, and more stable galvanometer recorder is adequate.

Results are expressed as in the previous paper.[6] From the record of the nitrogen meter and from the spirometer kymograph record of collected expired gas, a graph was prepared plotting the logarithm of 100 ($F_{A_{N_2}} - F_{I_{N_2}}$), which is the difference between end-expired nitrogen and inspired nitrogen (in the cylinder oxygen), expressed as per cent, on the ordinate against total expired volume (V_E) or against total alveolar volume (V_A) on the abscissa. The points on the graph represent nitrogen-volume values for frequently selected breaths during the entire period of oxygen breathing. This type of graph is our version of the so-called washout curve.

*The nitrogen meter, model A5, was manufactured by the Waters Company, Rochester, Minn. The recording milliammeter was made by the Esterline-Angus Company, Indianapolis, Ind.

†Previous experience at this laboratory has shown that functional residual capacity calculated by Van Slyke gas analysis of spirometer and alveolar samples usually differs from that calculated by simultaneous N_2 meter measurements by less than 100 ml.

RESULTS

The mean functional residual capacity of this group did not differ significantly from that in the previously studied normal young men ($P = 0.50$) (Table I). Similarly, the mean ratios of alveolar to total ventilation (V_A/V_E) were almost identical in the two groups (Table I). The values for recumbent functional residual capacity, vital capacity, total capacity, residual volume, and the ratio of residual volume to total capacity, listed in Table I, are comparable to those in older subjects studied by Kaltreider and associates[13] and Baldwin and associates,[14] but are lower in respect to functional residual capacity, residual volume, and the ratio of residual volume to total capacity than those of Greifenstein and co-workers,[15] whose subjects were all over 50 years old (mean age 61.5). There was no significant difference in mean functional residual capacity or residual volume/total capacity between or 10 subjects below age 50 and our 9 subjects over this age. The 80-year-old subject had lung volumes as follows: functional residual capacity 2.85 L., total capacity 5.75 L., vital capacity 3.95 L., residual volume 1.8 L., and residual volume/total capacity 0.314. These values fall well within the ranges of values for the entire group (Table I).

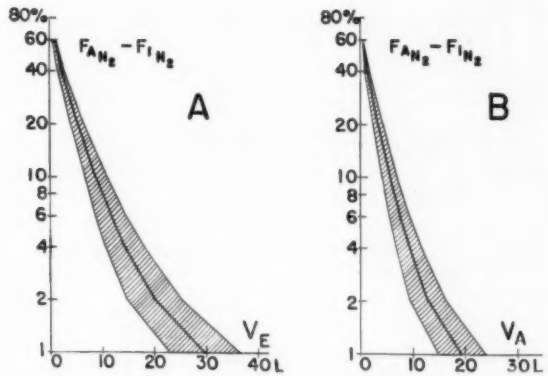

Fig. 1.—*A*, Course of decline of end-tidal nitrogen concentration to 1 per cent as function of total expired gas volume during oxygen breathing. *B*, The same as *A*, but relating decline in nitrogen concentration to alveolar ventilation. Heavy central lines represent mean values for 19 subjects. Cross-hatching represents one standard deviation from the mean. $F_{A_{N_2}} - F_{I_{N_2}}$ = per cent expired N_2 minus per cent inspired N_2.

The nitrogen washout curves which were obtained are analyzed as consisting primarily of two phases—an initial rapid decline of $F_{A_{N_2}}$ to the neighborhood of 1 per cent, and a subsequent slow decline to around 0.5 per cent. Tables II and III and Figs. 1, 2, and 3 summarize the data. As noted previously in the younger subjects, no constancy of number of slopes, nor of breaking points of the various slopes, nor of the terminal $F_{A_{N_2}}$ at the end of the test was found in the group as a whole. However, when repeated trials were made in selected subjects, the individual pattern of the washout curve in each person was remarkably similar.

TABLE I. CHARACTERISTICS OF NINETEEN OLDER NORMAL MEN

SUBJECT	AGE (YR.)	HEIGHT (IN.)	WEIGHT (LB.)	BODY SURFACE AREA ($M.^2$)	FUNCTIONAL RESIDUAL CAPACITY (L.)	RESIDUAL VOLUME (L.)	VITAL CAPACITY (L.)	TOTAL CAPACITY (L.)	RV/TC	TIDAL VOLUME (ML.)	$\dot{V}_E$ (L./MIN./$M.^2$)	V_A/V_E
1	43	67.5	165	1.87	2.09	1.55	4.45	6.00	0.259	602	3.73	0.628
2	47	64.5	151	1.75	1.97	1.39	3.21	4.60	0.302	993	5.43	0.687
3	50	65.5	150	1.76	1.57	1.22	2.98	4.20	0.291	650	5.22	0.600
4	46	68.0	145	1.79	2.69	1.78	4.36	6.14	0.290	675	3.83	--
5	48	67.5	135	1.72	3.06	2.26	3.58	5.84	0.387	701	4.78	0.608
6	38	70.5	135	1.78	2.22	1.47	4.68	6.15	0.239	685	4.51	0.677
7	48	65.0	155	1.78	1.44	1.15	3.90	5.05	0.228	910	3.58	0.731
8	44	73.0	187	2.10	3.62	2.72	5.43	8.15	0.334	817	5.50	0.627
9	50	69.5	148	1.83	3.67	2.18	4.47	6.65	0.328	876	5.06	0.644
10	37	69.0	146	1.81	2.34	1.17	4.98	6.15	0.190	648	4.24	0.657
11	56	66.5	178	1.91	1.41	1.07	3.62	4.69	0.229	1266	3.98	0.747
12	67	68.0	180	1.95	2.93	2.52	4.22	6.75	0.374	1487	4.79	--
13	54	71.0	165	1.94	1.89	1.39	4.85	6.24	0.223	621	3.52	0.696
14	54	70.0	180	2.00	2.03	1.26	3.99	5.25	0.241	789	4.50	0.692
15	53	69.0	154	1.84	2.44	1.68	5.08	6.76	0.248	802	4.54	0.658
16	56	67.0	155	1.82	2.70	1.80	4.37	6.17	0.292	977	4.07	0.725
17	58	69.0	150	1.83	2.79	1.71	4.44	6.15	0.278	1537	3.24	0.725
18	53	67.0	182	1.94	2.10	1.72	3.90	5.62	0.306	496	4.86	0.609
19	80	66.0	123	1.62	2.85	1.80	3.95	5.75	0.314	774	5.23	0.658
Mean	51.7	68.1	157	1.84	2.41	1.68	4.23	5.91	0.282	858	4.45	0.669
S.D.	9.9	2.2	18	0.10	0.66	0.47	0.63	0.91	0.052	289	0.68	0.046

RV/TC = Residual volume as fraction of total capacity.

$\dot{V}_E$ = Minute volume of expired gas, in liters per square meter of body surface area.

V_A/V_E = Alveolar fraction of total expired gas.

At the level of 1 per cent alveolar nitrogen ($F_{A_{N_2}} - F_{I_{N_2}}$), the mean summated expired gas volume, designated as V_E(1 per cent), and the alveolar fraction thereof, designated V_A (1 per cent), in these older subjects are significantly different from those in the previous younger group. For V_E(1 per cent) $P = 0.02$; for V_A(1 per cent) $P = 0.04$; for V_E(1 per cent)/FRC $P < 0.001$; for V_A(1 per cent)/FRC $P < 0.0001$ (Table II).* The coefficients of variation for these values are not greater in the older persons than in the younger.

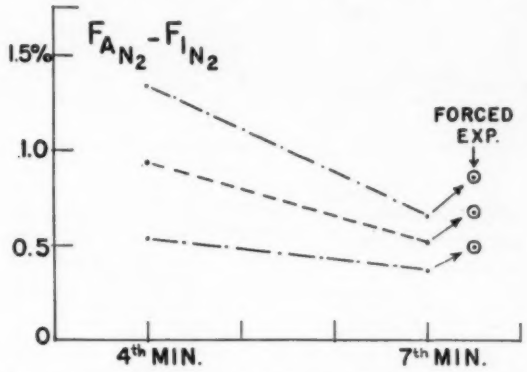

Fig. 2.—Schematic representation of decline of nitrogen concentration from 4 to 7 minutes of oxygen breathing, followed by a maximum expiration (19 subjects). Heavy dashed center line represents mean value. Outer dashed lines represent one standard deviation from the mean.

TABLE II. INITIAL OR STEEP PART OF CLEARANCE CURVE

$F_{A_{N_2}} - F_{I_{N_2}}$ (%)	V_E (LITERS)		V_A (LITERS)		V_E/FRC (LITERS)		V_A/FRC (LITERS)	
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Older Group—Present Study								
60	0.89	0.39	0.42	0.13	0.35	0.15	0.17	0.06
40	2.10	0.47	1.32	0.36	0.89	0.12	0.56	0.09
20	5.24	1.24	3.36	0.86	2.21	0.25	1.45	0.19
10	8.83	2.16	5.66	1.42	3.69	0.31	2.43	0.30
8	10.06	2.47	6.45	1.61	4.22	0.37	2.78	0.34
6	11.65	3.05	7.55	1.99	4.87	0.44	3.24	0.42
4	14.28	3.77	9.36	2.42	5.94	0.44	4.01	0.46
2	20.02	5.25	13.04	3.43	8.34	0.68	5.58	0.66
1	29.54	6.76	19.32	4.51	12.43	1.32	8.38	1.08
Younger Group—Previous Study								
1	25.14	5.70	16.55	4.46	10.97	1.29	7.08	0.89
$F_{A_{N_2}} - F_{I_{N_2}} = 1\%$								
P	0.02		0.04		< 0.001		< 0.0001	
Older Group—Present Study								
$\underline{V}$	22.9		23.4		10.6		12.9	
Younger Group—Previous Study								
$\underline{V}$	22.6		27.0		11.7		12.6	

Key: $F_{A_{N_2}} - F_{I_{N_2}}$ = per cent N_2 in expired alveolar gas minus per cent N_2 in inspired gas. V_E = total expired gas volume, or sum of tidal volumes. V_A = alveolar component of V_E. FRC = functional residual capacity. P = probability of random occurrence of such differences in means in these two groups. $\underline{V}$ = coefficient of variation.

*Based on the formula for significance of difference in means and Table XIV in Reference 16.

TABLE III. NITROGEN CLEARANCE IN LAST THREE MINUTES OF WASHOUT

	$F_{A_{N_2}} - F_{I_{N_2}}$ AT 4 MINUTES (%)	$F_{A_{N_2}} - F_{I_{N_2}}$ AT 7 MINUTES (%)	Δ' (%)	$F_{A_{N_2}} - F_{I_{N_2}}$ FINAL FORCED EXPIRATION (%)	Δ'' (%)
			Older Group—Present Study		
Mean	0.94	0.52	0.42	0.68	0.15
S.D.	0.40	0.14	0.32	0.18	0.10
			Younger Group—Previous Study		
Mean	0.72	0.47	0.25	0.53	0.06
S.D.	0.34	0.16	0.29	0.18	0.07
P	0.04	0.21	0.05	0.002	0.0002

Key: Δ' = difference between N_2 concentrations at end of 4 and 7 minutes. Δ'' = difference between N_2 concentrations produced by final forced expiration and the preceding ordinary breath at end of 7 minutes. P = probability of random occurrence of such differences in means.

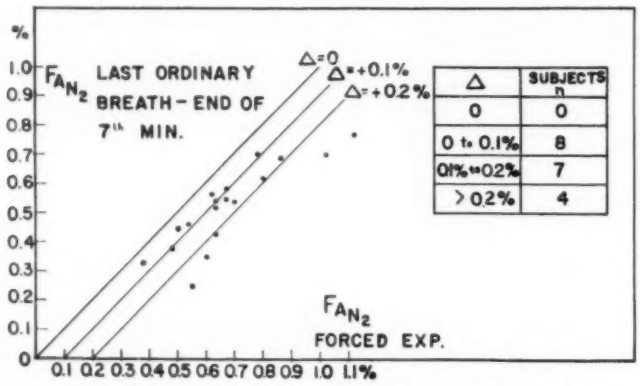

Fig. 3.—Difference between end-tidal nitrogen concentration of last tidal breath at end of 7 minutes of oxygen breathing and the nitrogen concentration produced by the terminal forced expiration (19 subjects).

Since the effect of variation in FRC can be eliminated by using the "turnover" ratios V_E(1 per cent)/FRC and V_A(1 per cent)/FRC,[6] the resultant larger ratios in the older subjects indicate impaired clearance efficiency or, in other words, impaired alveolar ventilation, presumably due to the presence of more numerous or larger poorly ventilated spaces than in the younger lungs.

The data for the slow part of the washout curves (values for $F_{A_{N_2}}$ less than 1 per cent) resemble those for the younger normal individuals previously studied, except that the final forced expiration produced a significantly greater increment of $F_{A_{N_2}}$ in the present group (Table III). The distribution of these increments is seen in Fig. 3. Whereas 33 per cent of the younger persons had shown no detectable rise in $F_{A_{N_2}}$ with the final forced expiration, each one of the older group produced some increment during this maneuver. Seventy-nine per cent of this group had no rise greater than 0.2 per cent; 4 individuals had rises greater than 0.2 per cent (0.3, 0.35, 0.32, and 0.25 per cent, respectively). The greater terminal rise in the older subjects, small as it is, reveals evidence of less uniform alveolar ventilation than in the younger lungs.

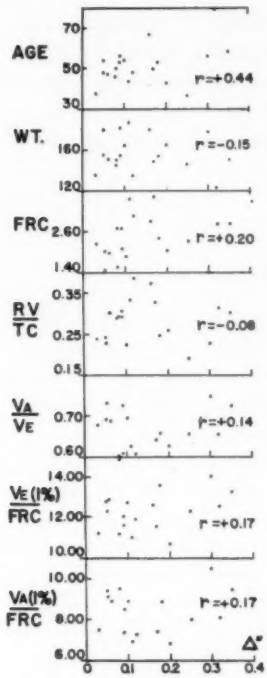

Fig. 4.—Relationship between alveolar nitrogen increment (Δ") produced by final forced expiration and various parameters. See text and previous illustrations for terminology. r = correlation coefficient.

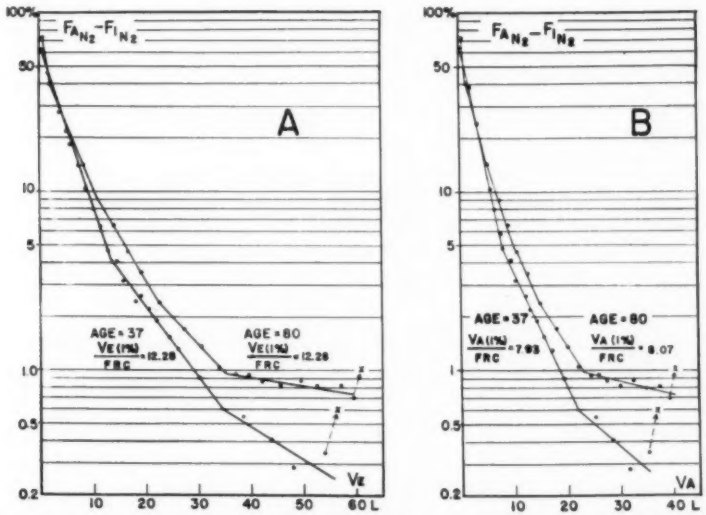

Fig. 5.—Nitrogen clearance curves of the youngest and the oldest subject in this study. X = F_{AN_2} of final forced expiration.

No obvious correlation could be found between the magnitude of the terminal rise in $F_{A_{N_2}}$ and age, body weight, lung volume, alveolar fraction of total ventilation, or the "turnover" ratios V_E(1 per cent)/FRC, and V_A(1 per cent)/FRC (Fig. 4).

Fig. 5 compares the washout curves of the youngest subject in this group and the oldest.

DISCUSSION

By a variety of methods, different from ours, previous investigators have found evidence for abnormal intrapulmonary gas mixing in middle and old age. Among those using nitrogen dilution techniques, Darling, Cournand, and Richards[17] detected impaired gas distribution in 3 normal men older than 50 years. Fowler, Cornish, and Kety,[8] with a nitrogen clearance method and an elaborate system of analysis of washout curves, concluded that there was uneven ventilation in 7 older normal people, due not only to increased FRC but also to actual presence of poorly ventilated spaces. Greifenstein's group[15] studied intrapulmonary gas mixing in 25 persons free of cardiopulmonary disease and over the age of 50. They compared two methods—the single breath nitrogen test of Fowler and Comroe[2] and the $F_{A_{N_2}}$ produced by a forced expiration at the end of a 7-minute period of oxygen breathing. The former test revealed abnormalities of gas mixing in 11 subjects. The latter test was abnormal in two subjects, with a mean $F_{A_{N_2}}$ of 1.57 per cent (S.D. 0.83) for the whole group which is in contrast to our mean value of 0.68 per cent (S.D. 0.18). In respect to techniques, our figure is corrected for inspired nitrogen in the cylinder oxygen, while no information on this point is given in their paper. Their criticism of the 7-minute test as less sensitive than the single breath test is directed at the final forced expiration nitrogen value as the sole criterion of completeness of mixing. Much more information than this can be obtained when the entire course of the nitrogen displacement from the lungs is followed breath by breath with the nitrogen meter.

Bates and Christie[18] found a mean helium mixing efficiency index of 54 per cent in 10 normal persons from 47 to 62 years of age, compared to an index of 76 per cent in 17 normals aged 17 to 37 years. Briscoe,[19] using a similar helium method, likewise found in 4 elderly normals (age 62 to 76 years) evidence of less uniform intrapulmonary mixing than in younger normal subjects. Needham and associates,[20] using similar methods, noted no greater impairment of mixing efficiency in older normal people (up to age 70) as compared to a younger group.

The terminal rise in $F_{A_{N_2}}$ with the final forced expiration implies the presence of alveolar spaces which are not ventilated as efficiently as the majority of alveoli. Correlation might be expected between this index of intrapulmonary mixing and the magnitude of V_E(1 per cent)/FRC and V_A(1 per cent)/FRC, since these ratios also measure efficiency of intrapulmonary mixing. Absence of such a relationship was found, and may be explained in two ways. First, the values for the turnover ratios require no special effort on the part of the

subject, merely that he keep his lips closed tightly about the mouthpiece during the period of oxygen breathing, which he may perform at any rate or depth which suits him. On the other hand, the terminal rise in $F_{A_{N_2}}$ will depend on the size of the final forced expiration, an effort requiring maximal cooperation. Thus the lack of correlation might be due to technical factors masking the true values for forced expiration $F_{A_{N_2}}$. Second, large values for V_E(1 per cent)/FRC and V_A(1 per cent)/FRC may measure a different set of poorly ventilated alveolar spaces than those revealed by the terminal $F_{A_{N_2}}$. This could imply a whole constellation of spaces with different modes of ventilation rather than simply one set of well-ventilated and another set of poorly ventilated alveoli. Evidence for the multiplicity of differently ventilated spaces exists in the shape of the curves in Figs. 5 and 6, where several different slopes are seen, a feature also noted in the previous paper.[6]

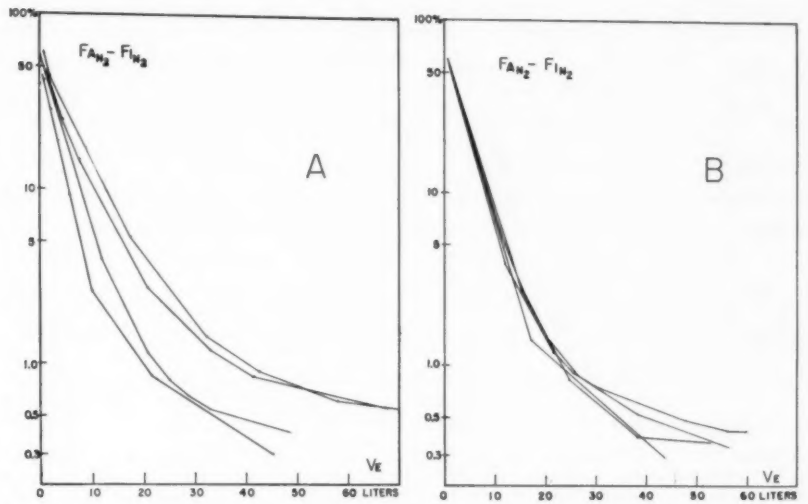

Fig. 6.—*A*, Washout curves of 4 different subjects, showing variations in slopes. *B*, Four washout curves from same subject, made over 21-month period.

SUMMARY

1. The course of displacement of intrapulmonary nitrogen while breathing oxygen for 7 minutes was followed breath by breath with a nitrogen meter in a group of 19 normal men ranging in age from 37 to 80 years. The qualitative and quantitative aspects of the washout curves were compared to data previously obtained in a younger group of normal men.

2. Evidence has been found for less efficient intrapulmonary gas mixing in the older group.

We are indebted to our various colleagues who served as experimental subjects and to Dr. David Salkin for helpful advice.

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CORRELATION BETWEEN ETHYL ALCOHOL LEVELS IN VENOUS BLOOD AND ALVEOLAR AIR SAMPLES

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THE diagnosis of drunkenness due to ethyl alcohol and the determination of the degree of alcoholic influence are usually functions of the local police agency. Qualified medical personnel are unavailable in most instances. Therefore, the degree of alcoholic influence has become a matter of subjective impression by police officers or other untrained individuals. This is a deplorable situation, especially when a reliable laboratory analysis would enable the courts to objectively assess the condition of the defendant.

This report presents a comparison of alcohol levels obtained by two analytic methods. One method employs a sample of venous blood and the other a sample of alveolar air.

It is the intent of this paper to demonstrate the validity of the relatively simple breath analysis method. There are a number of methods available for determination of blood alcohol levels,[1-3] and at least 4 devices are presently available for the determination of alcohol present in alveolar air.†

MATERIAL AND METHODS

In this study, a Harger Drunkometer[1] was employed in determining the alcohol level of alveolar air.

Blood alcohol levels were determined by the microdiffusion technique of Williams and associates.[3]

The blood samples studied were obtained from veins in the antecubital fossae of 3 male volunteers. Alveolar air was obtained from the lungs of the same 3 men. Care was taken to cleanse the skin at the needle puncture site with soap and water instead of alcohol, so as not to contaminate the blood sample. Separate dry sterile needles and syringes were used to obtain all blood specimens. Bourbon whiskey (86 proof) was given as the intoxicant.

RESULTS

Subject A had consumed 6 ounces of bourbon over a 2-hour period prior to initiating this study (Fig. 1). The initial alcohol level is recorded in Fig. 2.

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***Detective Inspector Langtry is Chief of the Scientific Bureau of the Detroit Police Department.

†The devices are: (1) Harger Drunkometer, Stephenson Corporation, Red Bank, N. J. (2) Breatholyzer, Stephenson Corporation, Red Bank, N. J. (3) Alcometer, Frederick G. Keyes, Inc., Cambridge, Mass. (4) Forrester Intoximeter, Intoximeter Association, 334 Buffalo Avenue, Niagara Falls, N. Y.

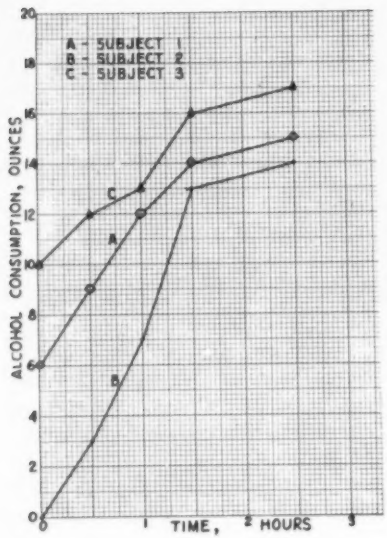

Fig. 1.

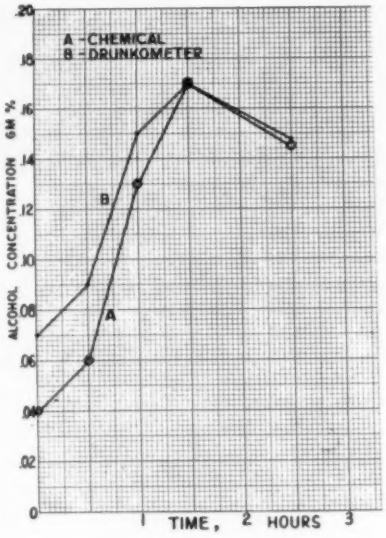

Fig. 2.

Fig. 1.—The amount of alcohol consumed and the period of time during which the study was conducted.
Fig. 2.—The blood and alveolar air determinations on Subject A.

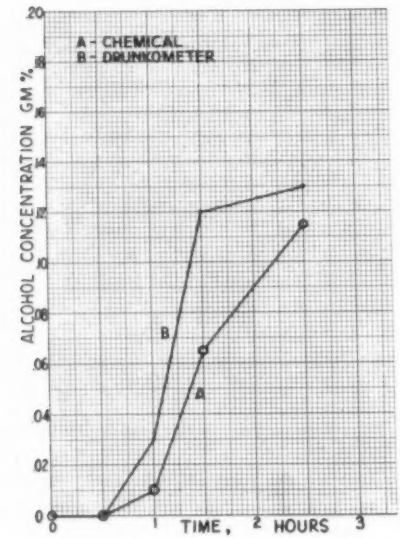

Fig. 3.

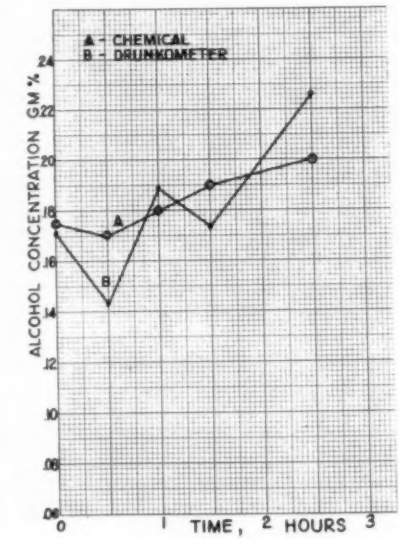

Fig. 4.

Fig. 3.—The blood and alveolar air determinations on Subject B.
Fig. 4.—The blood and alveolar air determinations on Subject C.

Over a period of 1½ hours, he consumed an additional 9 ounces of bourbon. A total of 15 ounces of whiskey was consumed within 2½ hours (Fig. 1). The alcohol levels present in the blood and alveolar air were measured initially and at 30-minute intervals. The determinations are illustrated by the graph in Fig. 2.

Subject B (Fig. 1) consumed no alcoholic beverage prior to the starting time. At this point, he was given 3 ounces of bourbon; at the end of 1½ hours he was given 11 ounces. At the end of the experiment (2½ hours), a total of 14 ounces had been consumed (Fig. 1). The blood and alveolar air alcohol levels are recorded in Fig. 3.

Subject C had consumed 10 ounces of bourbon over a 4-hour period prior to initiating this study. During the following 2½ hours, an additional 7 ounces of bourbon was consumed (Fig. 1). The alcohol levels are shown in Fig. 4.

DISCUSSION

No evidence of alcohol appeared in the blood or in the alveolar air of Subject B until at least 30 minutes after ingestion. When ingestion of the alcoholic beverage continued, the blood and alveolar air levels increased rapidly for a period, then both levels ceased to rise and were maintained. Thereafter, further imbibition caused another increase in both levels, although this last rise was considerably less rapid than the initial.

In our study, the levels obtained from the Harger Drunkometer were consistently higher than those obtained from venous blood. At levels less than 0.15 per cent, the differences are 1.1 to 1.4 times greater than those of the blood. At levels of 0.15 per cent and higher, the variation of the Drunkometer is 0.98 to 1.05 from the results of the blood determinations. It should be noted in Fig. 4 that the alveolar air level was considerably lower than the blood level at 30 minutes and 1½ hours. This anomaly in the curve was caused by the subject's ingestion of a quantity of water immediately before the Drunkometer test, thereby introducing a larger quantity of moisture into the system and causing adsorption of the alcohol on the inner surface of the balloon. This demonstrates one of the pitfalls which must be guarded against.

It is true that the Drunkometer test exhibited consistently higher levels of alcohol. It should be noted, however, that the blood levels were obtained on samples of venous blood; whereas, alveolar air contains levels of alcohol which closely approximate arterial blood levels. It is also to be noted that the height and weight of the individuals tested had no effect on the alcohol levels obtained by either method when comparable amounts of the intoxicant were ingested.

SUMMARY

A correlation between the Harger Drunkometer test of expired air for ethyl alcohol content and blood ethyl alcohol levels is presented. Also, the value and interpretation of these tests are discussed.

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ORNITHINE TRANSCARBAMYLASE ACTIVITY IN SERUM

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ENZYME levels estimated in serum have been used to detect hepatocellular damage. Glutamic oxalacetic transaminase,[1] glutamic pyruvic transaminase,[2] aldolase,[3] arginase,[4] lactic and malic dehydrogenases,[5] phosphohexoisomerase,[6] and isocitric dehydrogenase[7] serum concentrations have been found increased when liver cells are damaged. With the exception of arginase,[8] these enzymes are normally present in large concentration in tissues other than liver; therefore, it is often difficult to determine the affected organ from changes in serum activity of widely distributed enzymes.

Since ornithine transcarbamylase[9] is localized primarily in the liver[10] and since it can be estimated by a simple and reliable method,† we report here the assay method in detail and the measurements for this enzymatic activity in a number of sera from normal blood donors and from patients with hepatic diseases. In addition, the effect of experimentally produced liver damage on serum levels of ornithine transcarbamylase and transaminase was investigated together with a comparison of ornithine transcarbamylase and transaminase levels in sera of patients.

METHODS AND MATERIALS

The method is based on the colorimetric measurement of citrulline synthesized from carbamyl phosphate and ornithine by the catalytic action of ornithine transcarbamylase. The urea present in the serum, which would interfere with the colorimetric estimation of citrulline, is decomposed with urease; therefore, during the assay the synthesis of citrulline and the decomposition of the chromogenic urea proceed simultaneously.

Blood samples were collected in unoxalated tubes and the serum refrigerated until assay. Immediately before use 100 μM of dilithium carbamyl phosphate and 20 mg. of urease (type V Sigma, about 4 per cent pure) were dissolved in 10 ml. of 0.1 M phosphate buffer, pH 7.0; 1.0 ml. of this mixture, 1.0 ml. of 0.05 M L-ornithine, and 2.0 ml. of serum were mixed and incubated at 37° for 15 minutes. Tubes without ornithine or serum were used as blanks. Generally the blanks were negligible, although in some sera, as from uremic patients, it was necessary to increase the amount of urease or to preincubate the serum and urease for 15 minutes prior to the addition of the other reagents. The reaction mixtures were deproteinized by addition of 4.0 ml. of 1.0 N perchloric acid and centrifuged at 2,000 g for 10 minutes at 0° C. An aliquot of the supernatant fluid was analyzed for citrulline by the method of Archibald[12] with the following modifications. Aliquots containing no more than 0.8 μM

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†This work was presented at the Thirty-first Annual Meeting of the Central Society for Clinical Research and has been published in abstract form.[11]

of citrulline (usually 2.0 ml.) were made up to 5 ml., and 2 ml. of a 3:1 mixture of H_2SO_4 – H_3PO_4 (technical H_2SO_4 should be used), and 0.25 ml. diacetyl monoxime (3 per cent) was added. The contents were mixed and the tubes, capped with rubber stoppers fitted with capillary tubes, were heated in a covered, boiling water bath for 15 minutes. After cooling in the dark, the samples were read at 480 mμ. The color development deviates from Beer's law, hence a calibration curve is necessary. Transaminase activity was measured by a routine procedure.[18]

RESULTS AND DISCUSSION

In a previous study Manning and Grisolia[4] demonstrated that the estimation of arginase activity in serum offers a reliable and specific test for hepatocellular damage. This finding encouraged us to develop an even simpler method to measure another liver enzyme, ornithine transcarbamylase, an enzyme first discovered and partially purified from mammalian liver by Grisolia and Cohen.[9]

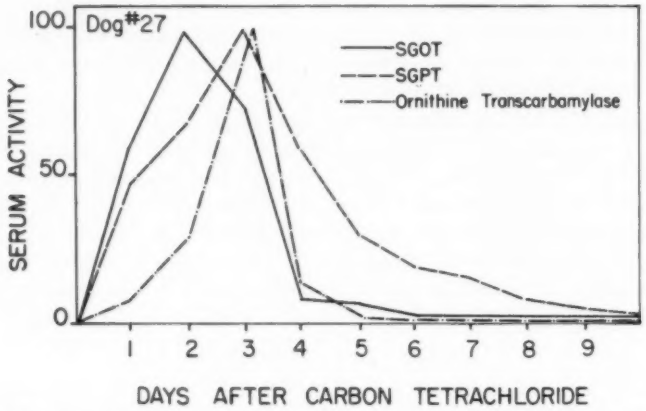

Fig. 1.—The increase of SGOT, SGPT, and ornithine transcarbamylase in dog serum following intragastric administration of 6 ml. CCl_4 per kilogram of body weight. Serum activities are expressed as per cent of maximum activity for each enzyme measured.

To determine the effect of hepatocellular damage of the serum activity of the enzyme, dogs were poisoned with carbon tetrachloride administered by means of a gastric tube according to Reissmann and associates.[14] This technique produces variable but definite hepatocellular injury and necrosis. The results of this procedure on the serum levels of ornithine transcarbamylase are shown in Fig. 1. For comparison, the activity for aspartic and glutamic transaminases (SGOT and SGPT) in the samples was determined and is also shown in the figure.

To establish normal values, sera from 32 blood donors and 38 hospitalized patients without clinical or laboratory evidence of liver disease were analyzed. Values ranged from 0.12 to 0.30 μM of citrulline per milliliter of serum under the assay conditions, with a mean value of 0.22 μM. The upper limit of normal was designated as 0.30 μM of citrulline per milliliter of serum. It is of interest that cerebrospinal fluid shows no activity. Bile obtained from the T tubes of postoperative patients has enzyme activity comparable with serum from the same patients.

The ornithine transcarbamylase activity in the serum of patients with various diseases of the liver is shown in Fig. 2, with elevations of enzyme activity in patients where hepatocellular damage or necrosis might be expected. In no case has an elevation of enzyme activity been observed which could not be attributed to a disease process involving the liver.

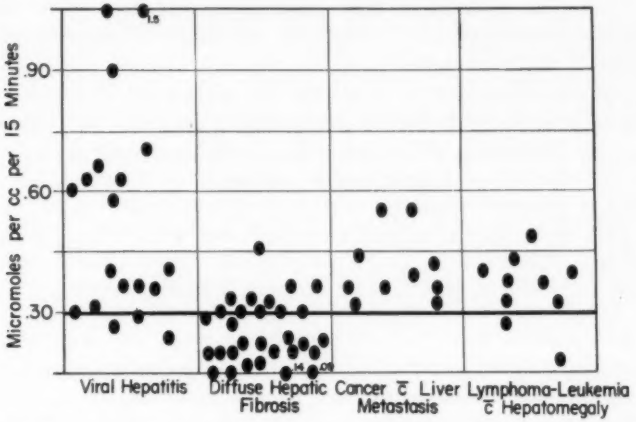

Fig. 2.—Serum ornithine transcarbamylase activity in patients with diseases involving the liver. Transcarbamylase activity is expressed in micromoles per milliliter of serum under the conditions of the assay.

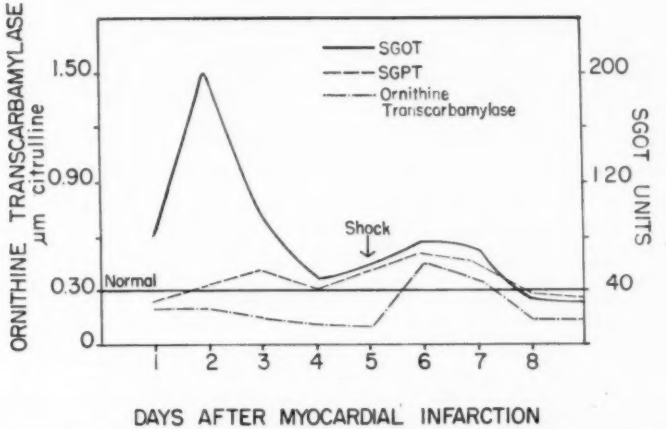

Fig. 3.—Serial daily determinations of SGOT, SGPT, and ornithine transcarbamylase in a patient with acute myocardial infarction. An uneventful postinfarction of 5 days was complicated by a gastrointestinal hemorrhage from a duodenal ulcer with hypotension of approximately 1 hour's duration but without electrocardiographic evidence of further infarction. The concomitant rise of ornithine transcarbamylase SGOT and SGPT reflects the common tissue source of the enzymes.

A few serial determinations on patients have been done. Fig. 3 shows the results of daily determinations on a patient with an acute myocardial infarction. Initial SGOT rise was accompanied by a slight elevation of the SGPT and no change in the ornithine transcarbamylase activity. The fifth postinfarction

day was complicated by a gastrointestinal hemorrhage and a prolonged episode of hypotension. Transient serum elevations of all 3 enzymes following this episode were attributed to hepatocellular damage resulting from shock.

Concurrent with the presentation of this method Reichard and Reichard presented their study on methods for estimation of ornithine transcarbamylase in serum.[15, 16] The methods of these authors are based upon the arsenolysis of citrulline and of carbamyl C^{14} citrulline and on the measurements of $C^{14}O_2$ and/or ammonia to follow activity.

These authors discussed at length the rationality of using the arsenolysis of citrulline to measure ornithine transcarbamylase activity. Reichard and associates have confirmed with extensively purified ornithine transcarbamylase the work of Grisolia and Cohen[9] and of Grisolia and Marshall,[10] particularly the identity of ornithine transcarbamylase with the enzyme arsenolyzing citrulline.[10, 17] We feel that the method presented here is simpler and more rapid and requires equipment available in most routine clinical laboratories. On the other hand, the isotopic method may be more sensitive.

SUMMARY

1. A rapid, simple assay for the estimation of the enzyme ornithine transcarbamylase in serum is presented.

2. The serum activity of ornithine transcarbamylase is increased specifically as a result of hepatocellular damage.

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LABORATORY METHODS

A SURVEY ON THE APPLICATION OF VISUAL INDICATORS FOR THE CHELOMETRIC CALCIUM DETERMINATION IN SERUM

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THERE is need in clinical analysis for simple, rapid, yet relatively accurate methods for calcium and magnesium estimation. In the last 40 years, there has been a tremendous increase in literature concerning chemical methods for the quantitative determination of calcium in serum and biologic fluids. The methods described have been based on precipitation of calcium from biologic solution as its insoluble oxalate or phosphate, the calcium being determined either by direct analysis[1, 2] or indirectly by analysis of the anion.[3-8]

The classical indirect method of Clark and Collip,[5] based on the precipitation as oxalate, centrifugation, washing of the precipitate, and titration with potassium permanganate, is a complicated and somewhat inaccurate procedure. As a rule, modification of the original procedure, e.g., by using cerium sulfate instead of potassium permanganate, does not produce any real improvement. The extent of the inaccuracy has been shown by the Belk and Sunderman survey.[9] These authors report a range of 7.0 to 26.6 mg. per cent on a calcium specimen of 12.6 mg. per cent, and of the laboratories surveyed 30 out of 44 reported results outside the accepted variation range of ± 0.5 mg. per cent. The other disadvantages of the precipitation methods are that they are time consuming (requiring a period of 2 to 24 hours), an inconvenience in the treatment of hypoparathyroid tetany, and require more serum than can be obtained from pediatric patients.

The direct determination of calcium in biologic fluids with flame photometers[10-11] is a difficult task for most small clinical laboratories. Colorimetric methods[2-12, 13] are either time consuming or need careful handling and special precautions.

In an effort to find a method which can obviate these difficulties, many authors are now proposing methods for calcium and calcium plus magnesium in blood serum based on the chelometric principle. The most outstanding chelating reagent which is widely applied for the quantitative determination

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of these metal ions is ethylenediaminetetraacetic acid (EDTA or H_4Y) which forms stable stoichiometric 1:1 complexes involving the amino-acetic acid group in EDTA.[14]

After the introduction of metallochromic indicators by Schwarzenbach and co-workers,[15] the possibility of using EDTA as a titrant for calcium or calcium and magnesium was explored. EDTA is an unselective chelon and forms complexes with both calcium and magnesium. Sobel and Hanok[16] developed an indirect procedure for determining the sum of Ca plus Mg using EDTA as titrant and Eriochrome Black T as the indicator. The titration of calcium alone in a mixture of Ca plus Mg proved to be difficult because at pH values higher than 11 Eriochrome Black T does not function as suitable indicator; yet, magnesium hydroxide precipitates quantitatively only at pH higher than 12.

Most authors have resorted to the use of oxalate precipitation of calcium from deproteinized[17, 18] or native pooled serum[19-22] Some authors and workers in clinical laboratories have expressed dissatisfaction with the existing procedures involving precipitation for two reasons: (1) The calcium end point with the dye Eriochrome Black T as a whole is not satisfactory. (2) The end point drags due to the back precipitation of calcium in the presence of alkaline oxalate in solution.

Several authors have tried to overcome these difficulties. Flaschka and Holasek[17] add an excess of stoichiometric magnesium-EDTA to the ammoniacal calcium solution before adding the indicator, thus replacing the poor calcium end point with the good one of magnesium. Nielsen[23] dissolved the calcium oxalate precipitated in a known amount of EDTA and back titrated the excess with standard magnesium solution at pH 10.0. Kimbel[19] improved the end point by adding a drop of a yellow dye (methyl red) and by greatly diluting his sample. Piette and Piette[24] also used methyl red as a screening dye, and Gerlach[25] used methyl orange. A brochure by Merck[21] recommends the use of a mixed indicator in tablets formed of Eriochrome Black T, a yellow azo dye, ZnY^{--}, and ammonium chloride. Grette[26] decomposed the oxalic acid with perchloric acid and used Eriochrome Black T. Schulitz[27] used cresolphthalein complexion instead of Eriochrome T and added MgY^{--} and methyl red to improve the end point. He decomposed the oxalic acid with a bromide-bromate sulfuric acid mixture; this procedure, however, is long, complicated, and time consuming.

All authors are enthusiastic about the titration of calcium and/or magnesium with EDTA and all agree that chelometric methods are superior in accuracy to the classical analytic methods. The superiority of the chelometric methods was shown recently.[29] Artificial serum, containing all the component of serum, was prepared and analyzed by experienced workers using both classical and chelometric methods. The results of 20 determinations with each method are recalculated and represented by the average values and standard deviation in Table I.

It is clearly shown in Table I that the chelometric methods give more accurate and reproducible results. By the classical method the possibility of

error is greater because calcium oxalate usually occludes alkali oxalate in the precipitate giving rise to a positive error. The negative error is attributed to the solubility of calcium oxalate and the consequent loss during washing. Each washing with 5 ml. ammoniacal solution results in a loss of 0.3 to 0.6 mg. per cent of calcium. It is clear that the inaccuracy due to occluded alkali oxalate is not present in the chelometric calcium estimation; but the negative error due to the solubility of calcium oxalate still exists. During the precipitation step the serum is diluted to 2 or 3 times its volume, giving rise to a negative error of 0.2 to 0.5 mg. per cent.

TABLE I. CALCIUM DETERMINATION IN ARTIFICIAL SERUM CONTAINING 10.00 MG. PER CENT CALCIUM CALCULATED FROM PUBLISHED RESULTS[29]

METHOD	HIGHEST VALUE	LOWEST VALUE	AVERAGE VALUE (20 DETERMINATIONS)	STANDARD DEVIATION
Permanganate	11.5	9.3	10.32	0.16
Ceric sulfate	11.0	9.2	10.25	0.13
EDTA	10.5	9.6	10.06	0.05

A further error observed by several workers[18, 30, 31] as well as the authors is caused by using aliquots from deproteinized serum. (The supernatant liquid over the precipitated protein does not correspond to the whole volume before precipitation.) Calcium errors as high as 4 per cent due to this cause have been reported.

All the available chelometric methods which employ precipitation of calcium as oxalate are time consuming and all have the faults inherent in precipitation methods. These faults could be eliminated by titrating the calcium directly in blood serum or biologic fluids by using a specific calcium indicator. This requirement is established by the introduction of murexide (acid ammonium purpurate) as the specific indicator for the direct chelometric determination of calcium at pH values higher than 12. Greenblatt and Hartmann,[32] Buckley and co-workers,[33] Holtz,[34-36] Elliot,[37] and Gautier and associates[38] developed visual titration methods for serum using ammonium purpurate as the indicator for calcium estimation. The visual end point of this indicator in serum, however, is indistinct and easily obscured by hemolysis and bilirubinemia. To overcome these difficulties Kibrick and associates,[39] Fales,[40] Lehman,[41] and Horner[42] adapted the chelometric procedures of serum for photometric measurements. Recently Golby and associates[43] and Flaschka and co-workers[44] introduced other specific calcium indicators for serum analysis.

Because many biochemists are no longer satisfied with the accuracy of visual indicators, we carried out a survey of the relative merits of the indicators presently available for the direct determination of calcium in serum. Of the 8 different indicators studied, only 3 (murexide, Eriochrome Blue Black R, and Eriochrome Blue SE) had previously been used in serum titrations.

MATERIALS AND METHODS

Before a comparison of the indicators was attempted, the degree of interference of various species present in native serum and of other impurities likely to be present in the

reagents employed was studied intensively. Studies were carried out with pure solutions to which the impurity under investigation had been added.

Phosphate Anion.—The presence of phosphate ion was found to have no effect when it was present in the proportions found in serum. If, however, the proportion of phosphorous to calcium is greater than 2:1, interference is observed, and better results are obtained by back titration.

Copper.—Copper was found to be the most undesirable impurity present as it blocks most of the indicators used. By employing the highest grade chemicals and masking traces of copper with potassium cyanide, the effect of this impurity can be eliminated.

Iron and Manganese.—These are also undesirable impurities, but their effect can be abolished by reducing them to the lower valency state with ascorbic acid in neutral solution, and subsequently masking the traces of iron with cyanide.

Magnesium.—This has no effect on the calcium titration as long as its concentration does not exceed that of the calcium, and a controlled pH of approximately 12.5 to 12.7 is maintained so that the magnesium is masked by precipitation. Precipitation commences at pH 10.5 and some ions remain in solution at pH levels below 12.3 giving a reddish tinge to the indicator.

After studying the effects of interfering ions and their removal, a general procedure was established to compare the quality of the end point with various indicators on the same type of homogeneous human pooled serum.

Treatment of Glassware.—Glassware used in calcium determination must be of Pyrex and pretreated to decrease the effect of the ion exchange property of the glass walls and to remove any reactive metal ions present on the surface of the glass. Erlenmeyer flasks were pretreated with hot dilute nitric acid and, after rinsing with water and concentrated alkaline EDTA solution (about 5 per cent) heated to boiling, are left overnight and finally rinsed several times with demineralized water. The EDTA burette and volumetric flask were filled with concentrated alkaline EDTA for two days after the acid treatment (chromic-sulfuric acid mixture), then rinsed several times with demineralized water.

Reagents and Solutions.—All chemicals used in this investigation were of reagent grade quality unless otherwise stated.

Demineralized water: The usual laboratory distilled water is passed through a Barnstead Bantam demineralizer. Only this water is used for preparing solutions or diluting the serum.

EDTA solution 0.001 M: The solid dihydrate of ethylenediaminetetraacetate is dried several days in a vacuum desiccator or for 2 hours at 80° C. in a drying furnace. 3.725 Gm. are weighed and diluted to 1 L. in a volumetric flask pretreated as stated above. Immediately after preparation the solution is transferred to a polyethylene bottle. The accuracy of the titrant was checked by titrating with a standard zinc solution using Eriochrome Black T (F241-CI203) and pH 10 buffer.

Standard 0.01 M zinc solution: Prepared either from zinc metal or from zinc oxide (which has been heated several hours in a muffle furnace at 800° C.) by dissolving in a sufficient quantity of dilute nitric acid. A solution of 0.001 M is prepared by dilution.

Standard calcium solution: Primary standard calcium carbonate is dried in an oven overnight at 110° C. The appropriate amount to prepare 0.01 M is weighed and dissolved in dilute nitric acid.

Buffer pH 10: Ammonium chloride, 13.5 Gm., is dissolved in 100 ml. demineralized water, and 88 ml. fresh concentrated aqueous ammonia is added. The solution is diluted to 250 ml., and the pH is checked with a pH meter. The solution is kept in a polyethylene bottle.

Sodium hydroxide 50 per cent (carbonate-free): Sodium hydroxide, 125 Gm., is dissolved in 125 ml. demineralized water. The solution is stored in a polyethylene bottle. (Commercial 50 per cent sodium hydroxide is also suitable.)

Ascorbic acid: Solid.

Potassium cyanide: Keep in a well-capped bottle.

Buffer pH 12.5: Diethylamine hydrochloride, 7.5 Gm., is dissolved in 200 ml. water and the pH is adjusted with a pH meter to 12.7 by adding sodium hydroxide. The solution is diluted to 250 ml., and stored in a polyethylene bottle.

Indicators: The commercially available indicators were used in solid form to reduce oxidation and deterioration.

a. Eriochrome Black T (F241-CI203, J. T. Baker Chemical Company) is finely ground with sodium chloride in the proportion 1:400 and is used with buffer pH 10 to standardize the EDTA solution against standard zinc solution.

b. Murexide (ammonium purpurate, J. T. Baker Chemical Company) is finely ground with sodium chloride in the proportion 1:400.

c. Calcein (G. F. Smith Chemical Company), is finely ground with sodium chloride in the proportion 1:400.

d. Patton and Reeder dye (HHSNN, Fisher Scientific Company) is finely ground with sodium chloride in the proportion 1:400.

e. Calcochrome Blue Black BC (CI201, American Cyanamid Company): 0.1 Gm. solid indicator is mixed with 0.14 Gm. naphthol green B and finely ground with 20 Gm. sodium chloride.

f. Eriochrome Blue Black R (F240-CI202, Calcon, J. T. Baker Chemical Company) is finely ground with sodium chloride in the proportion 1:400.

g. Eriochrome Blue SE (Geigy, Basel): 0.1 Gm. solid indicator is mixed with 0.17 Gm. naphthol green B and finely ground with 20 Gm. sodium chloride.

h. Methylthymol blue (Chemapol, Prague, Czechoslovakia) is finely ground with sodium chloride in the proportion 1:400.

i. Calcofast Blue 2G, Pr 144 (unmetallized) (American Cyanamid Company) is finely ground with sodium chloride in the proportion 1:400.

Procedure.—

A. *For direct estimation of calcium in serum:* One milliliter of serum is pipetted into a 50 ml. Erlenmeyer flask, and 30 ml. of demineralized water and 2 mg. of ascorbic acid are added. After stirring, 5 mg. potassium cyanide is added followed by 3 drops of 50 per cent sodium hydroxide (carbonate-free). The pH of the solution must not be higher than 12.7 or lower than 12.5 and it is recommended that 2 ml. of the diethylamine buffer be added. The solution is mixed well and an appropriate amount of indicator is added. The calcium is titrated with 0.001 M EDTA solution to the clear stable color of the overtitrated comparison solution.

Calculation:

$$\text{ml. EDTA} \times \text{molarity} \times 4.008 = \text{mg. Ca per 100 ml. serum.}$$

B. *Back titration of calcium in serum:* One milliliter of serum is pipetted into a 50 ml. flask, followed by 25 ml. demineralized water and 2 mg. ascorbic acid. After stirring, 5 mg. of potassium cyanide is added and the solution is well shaken. Three drops of 50 per cent NaOH, 2 ml. diethylamine buffer, and 5.00 ml. of 0.001 M EDTA are added, followed by the indicator, and the excess free EDTA is titrated with the standard calcium solution to a clear stable color of the calcium dye complex as shown by a comparison solution.

Calculation:

$$\text{ml. EDTA} \times \text{molarity} - \text{ml. Ca} \times \text{molarity} \times 4.008 = \text{mg. Ca per 100 ml. serum.}$$

RESULTS AND DISCUSSION

The results obtained for the direct analysis of calcium ion in native serum using different calcium metallochromic indicators are given in Table II. The

accepted value for calcium for the applied homogeneous serum was determined by titration with EDTA after careful precipitation as oxalate from native serum.* With each indicator a series of 6 determinations were carried out by 3 different workers. The average values and the standard deviation of each worker as well as the mean of all workers are presented in Table II. From the table it is obvious that the average values of each worker and the mean of the 3 workers using murexide and calcein are considerably lower than the amount of calcium actually present. The color change with Patton and Reeder dye is quite sharp but does not occur at the actual equivalence point. From these results we can say that these 3 indicators are not to be recommended for visual titrations. The remaining 5 indicators are of the same over-all quality in the accuracy and reproducibility of the accepted error in clinical methods with the trained workers A and C. Worker B was less experienced in visual titrations and the lower results given may also be caused by weaker color perception.

TABLE II. DIRECT DETERMINATION OF CALCIUM IN NATIVE SERUM (9.86 MG. PER CENT)

INDICATOR	AVERAGE VALUE (6 DETERMINATIONS)				STANDARD DEVIATION			
	A	B	C	MEAN	A	B	C	MEAN
Murexide	8.86	9.11	8.86	8.94	0.75	0.49	0.56	0.59
Calcein	9.12	8.91	9.19	9.07	0.70	0.85	0.37	1.06
Patton and Reeder dye	9.50	9.58	9.21	9.43	0.18	0.34	0.27	0.12
Eriochrome B 201	9.93	9.54	9.82	9.76	0.39	0.62	0.22	0.45
Eriochrome R 202	9.87	9.89	9.92	9.91	0.22	0.31	0.30	0.27
Eriochrome SE	9.83	9.63	9.80	9.75	0.21	0.27	0.10	0.21
Methylthymol blue	9.82	9.51	9.99	9.77	0.13	0.21	0.16	0.26
Calcofast Blue 2G	10.15	9.56	10.19	9.96	0.19	0.04	0.25	0.34

TABLE III. DIRECT DETERMINATION OF CALCIUM IN DEPROTEINIZED SERUM (10.05 MG. PER CENT)

INDICATOR	AVERAGE VALUE (6 DETERMINATIONS)				STANDARD DEVIATION			
	A	B	C	MEAN	A	B	C	MEAN
Murexide	9.33	9.75	9.31	9.46	0.29	0.43	0.32	0.39
Calcein	10.24	9.29	9.36	9.61	0.38	0.31	0.31	1.16
Patton and Reeder dye	9.65	10.20	9.68	9.84	0.19	0.51	0.18	0.41
Eriochrome B 201	10.10	9.71	10.13	9.98	0.12	0.21	0.09	0.24
Eriochrome R 202	10.21	9.82	10.30	10.22	0.19	0.21	0.14	0.35
Eriochrome SE	10.01	9.41	9.95	9.79	0.23	0.27	0.23	0.36
Methylthymol blue	9.80	9.80	10.09	9.89	0.19	0.21	0.16	0.23
Calcofast Blue 2G	10.09	10.05	10.01	10.05	0.29	0.19	0.13	0.21

Table III shows some typical results for the analysis of calcium ion in deproteinized serum using the indicators. The accepted value was determined by titration with EDTA after precipitation as oxalate using Eriochrome Black T as indicator and MgY^{--} as indicator sensitizer. The results represented with the first 3 indicators are clearly outside the variation range accepted in clinical analysis. The results of the other 5 indicators coincide with those given in Table II and are no better than the results of the direct titration in

*According to Flaschka and Holasek method[17] but without diluting the serum.

native serum.* An interesting feature here is the clear difference in calcium value of the deproteinized and native serum. (The same sample of homogeneous serum was used for each.) This shows that direct titration of calcium in native serum gives more reliable results.

The results in Table IV are mean values of duplicate determinations by the same procedure for 6 serum samples to which 10.00 mg. calcium per 100 ml. serum have been added. The average recovery of added calcium is 10.08 mg. for Calcochrome Blue Black BC (CI 201), 10.07 mg. for methylthymol blue, and 10.05 mg. calcium per 100 ml. of serum for Calcofast Blue 2G.

TABLE IV. RECOVERY OF 10.00 MG. CALCIUM ADDED TO SERUM

	CALCIUM (MG. PER 100 ML. SERUM)			
		CALCIUM RECOVERED		
SAMPLE NO.	SERUM	ERIOCHROME B 201	METHYLTHYMOL BLUE	CALCOFAST BLUE 2G
1	9.86	10.00	10.04	10.04
2	10.36	10.04	10.12	10.24
3	8.76	10.32	10.24	10.20
4	12.20	10.14	10.12	9.92
5	9.00	9.96	9.96	10.00
6	11.08	10.04	9.94	9.92

The last 5 indicators can be divided into 2 groups; the first includes Eriochrome Blue Black R CI202, Eriochrome Blue SE, and Calcofast Blue 2G which have a color change from red to clear stable blue, and the second includes Calcochrome Blue Black BC CI 201 which changes color from red to colorless or gray and methylthymol blue which changes color from blue to gray or colorless according to the amount of indicator added.

For those workers who cannot easily differentiate between the slight changes in color shades or whose color perception is weak in reddish colors, the authors recommend the second group of indicators whose color change is to colorless or grayish. The use of a color comparison (solution titrated beyond the end point) is often advisable in routine work. The worker must familiarize himself with the character of the color change taking place at the end point before carrying out routine determinations.

More accurate and reproducible results for calcium may be obtained by back titration with a standard calcium solution. The procedure here is the same as the direct one except that a known excess of standard EDTA solution is added, the solution is back titrated with a standard calcium solution to the color of the calcium indicator complex, and finally with EDTA to the clear stable color of the overtitrated comparison solution.

Superior results are sometimes obtained by replacing EDTA with ethylene glycol bis-(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA)[45] and using the same recommended indicators.

*The end points using methylthymol blue were indistinct but by adding 2 drops of 0.1 per cent methyl red and 1 drop of 0.1 per cent naphthol green B fairly good end points were obtained as represented in Table III.

SUMMARY

Statistical comparison of calcium values of an artificially prepared serum shows the efficiency of the chelometric oxalate method in comparison with the method of Clark and Collip or its modification. The classical approach and the more laborious oxalate precipitation seem to be undesirable. Furthermore, the direct chelometric methods have the advantage of being able to give the calcium estimation for a particular sample within 2 to 5 minutes.

A critical survey has been made with the available visual metallochromic calcium indicators to determine calcium in human pooled serum. For routine work the direct chelometric determination of calcium deserves consideration for its accuracy and applicability.

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FURTHER INVESTIGATION ON THE SELECTIVE ACTIVITY OF FLUID THIOGLYCOLLATE MEDIUM FOR GROUP DIFFERENTIATION OF M. TUBERCULOSIS, ANONYMOUS (ATYPICAL) ACID-FAST BACILLI, AND SAPROPHYTIC MYCOBACTERIA

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INTEREST in pulmonary disease and other human infections caused by atypical mycobacteria has been greatly intensified in recent years. Because such organisms are often difficult to distinguish from tubercle bacilli and other acid-fast bacteria, investigators have been developing procedures which are aiding in the identification of these organisms.

In a previous study[1] it was observed that fluid thioglycollate medium was useful for group differentiation of *Mycobacterium tuberculosis,* atypical acid-fast bacilli, and saprophytic mycobacteria. This observation was confirmed by Koch and associates.[2]

The present investigation was undertaken to determine further the differential potentiality of the fluid thioglycollate medium. In addition, all the organisms were tested for their capacity to bind neutral red, to produce catalase and peroxidase, and to form "serpentine cords."

MATERIALS AND METHODS

Fifty strains of freshly isolated human tubercle bacilli, 50 strains of atypical acid-fast bacilli, and 5 strains of saprophytic mycobacteria were selected for study. The majority of the organisms were supplied through the courtesy of other workers, as indicated.

Tubercle Bacilli.—Strains A-1 through A-22 were obtained from Mr. Frank Clifton, Veterans Administration Hospital, Alexandria, Louisiana. Strains W-1 and W-2 were obtained from Miss Gloria E. Veach, Veterans Administration Center, Wadsworth, Kansas. Strain H37Rv was obtained from Dr. William Steenken, Jr., Trudeau Laboratory, Saranac Lake, New York.

Atypical Mycobacteria.—Strains AP-8 and AS-10 through AS-13 were obtained from Mr. Frank Clifton. Strains UP-GI-P-18, UP-GI-P-21, US-GII-P-6, and US-GII-P-19 were obtained from Dr. Ernest H. Runyon, Veterans Administration Hospital, Salt Lake City, Utah.

Saprophytic Mycobacteria.—Strains *M. smegmatis*-a, b, *M. phlei*-a, and *M. butyricum* were obtained from Dr. George P. Kubica, Communicable Disease Center, Public Health Service, Chamblee, Georgia. Strain *M. phlei*-b was obtained from Dr. William Steenken, Jr.

The tubercle bacilli and atypical strains were isolated from various clinical specimens, such as sputa, bronchial washings, gastric lavages, resected lung lesions, pleural

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fluids, lymph nodes, and catheterized ureteral urines. Of the 50 atypical strains, 20 were photochromogens, and 30 were scotochromogens. To avoid the use of patients' names and for convenience in arrangement, most of the strains have been redesignated.

All the organisms were transferred to glycerol-blood agar,[3] Löwenstein-Jensen medium,[4] oleic acid-albumin agar,[5] and Proskauer and Beck[6] medium to maintain stock cultures; the strains were removed subsequently for use in the various tests. Each organism was grown in Tween albumin medium[7] for 14 days, and then inoculated into two tubes of fluid thioglycollate medium* with 4 mm. inoculating loops using two loopfuls per tube. All tubes were incubated at 37° C. and readings were made daily for a period of 8 weeks. Smear preparations were stained by the Ziehl-Neelsen technique. The catalase, peroxidase, neutral red, and serpentine cord tests were performed by the methods described elsewhere.[6, 8-10] All tests were repeated 3 times. The final readings represent average results.

RESULTS

The data presented in Table I summarize typical findings from each group of organisms. The thioglycollate medium failed to support the growth of any of the tubercle bacilli, but did support the growth of 84 per cent of the atypical strains (7 photochromogens and 14 scotochromogens) and 100 per cent of the saprophytes. Growth of the atypical mycobacteria was slow and dysgonic. The saprophytes grew rapidly and luxuriantly in one day with pellicle formation. Eleven to twenty-eight days were required for initial growth of the photochromogens and 12 to 39 days for the scotochromogens.

Every organism of each group gave a positive catalase reaction which was most pronounced among the photochromogens and scotochromogens. All of the tubercle bacilli gave positive results with each of the 3 other tests, whereas all of the atypical and saprophytic strains, with two exceptions, gave negative results. Slight to moderate degrees of cord formation were exhibited by two of the saprophytic organisms. However, this was only observed in portions of the smears. The majority of the bacilli were disoriented and formed amorphous clumps which is characteristic of avirulent mycobacteria. The ability of saprophytic acid-fast bacilli to form serpentine cords had been reported previously.[11, 12]

DISCUSSION

Although the peroxidase, neutral red, and cord tests can distinguish tubercle bacilli from atypical and saprophytic mycobacteria, they are not able to differentiate the atypical from the saprophytic groups of organisms. On the other hand, in most instances, the thioglycollate test is able to distinguish all 3 groups, and when used in conjunction with the other tests, differentiation is further facilitated. In addition to the atypical and saprophytic mycobacteria, all isoniazid-resistant tubercle bacilli are also deficient in the enzyme peroxidase. Therefore, such tubercle bacilli will give negative or weakly positive peroxidase reactions depending upon the degree of drug resistance. Since catalase was produced by all the organisms, this test is of no differential value under these conditions.

*Thioglycollate medium without indicator, Brewer modified, Baltimore Biological Laboratory, Baltimore, Md., No. 01-135-C, Lot Numbers 9803 and 10881.

TABLE I. GROWTH OF M. TUBERCULOSIS, ATYPICAL ACID-FAST BACILLI, AND SAPROPHYTIC MYCOBACTERIA IN THIOGLYCOLLATE MEDIUM, AND COMPARATIVE RESULTS OF THEIR CATALASE, PEROXIDASE, NEUTRAL RED, AND SERPENTINE CORD REACTIONS

STRAIN	GROWTH IN THIOGLY-COLLATE MEDIUM	CATALASE REACTION	PEROXIDASE REACTION	NEUTRAL RED REACTION	SERPENTINE CORD FORMATION
Human tubercle bacilli					
A-1	–	+	+	+	+
A-2	–	+	+	+	+
A-3	–	+	+	+	+
A-4	–	+	+	+	+
A-5	–	+	+	+	+
A-6	–	+	+	+	+
A-7	–	+	+	+	+
A-8	–	+	+	+	+
A-9	–	+	+	+	+
A-10	–	+	+	+	+
A-11	–	+	+	+	+
A-12	–	+	+	+	+
A-13	–	+	+	+	+
A-14	–	+	+	+	+
A-15	–	+	+	+	+
A-16	–	+	+	+	+
A-17	–	+	+	+	+
A-18	–	+	+	+	+
A-19	–	+	+	+	+
A-20	–	+	+	+	+
A-21	–	+	+	+	+
A-22	–	+	+	+	+
W-1	–	+	+	+	+
W-2	–	+	+	+	+
H37Rv	–	+	+	+	+
Atypical mycobacteria photochromogens					
AP-1	28+	+	–	–	–
AP-2	27+	+	–	–	–
AP-3	26+	+	–	–	–
AP-4	25+	+	–	–	–
AP-5	23+	+	–	–	–
AP-6	19+	+	–	–	–
AP-7	11+	+	–	–	–
AP-8	–	+	–	–	–
UP-GI-P-18	–	+	–	–	–
UP-GI-P-21	–	+	–	–	–
Atypical mycobacteria scotochromogens					
AS-1	39+	+	–	–	–
AS-2	28+	+	–	–	–
AS-3	27+	+	–	–	–
AS-4	27+	+	–	–	–
AS-5	26+	+	–	–	–
AS-6	25+	+	–	–	–
AS-7	25+	+	–	–	–
AS-8	24+	+	–	–	–
AS-9	22+	+	–	–	–
AS-10	20+	+	–	–	–
AS-11	14+	+	–	–	–
AS-12	13+	+	–	–	–
AS-13	–	+	–	–	–
US-GII-P-6	26+	+	–	–	–
US-GII-P-19	12+	+	–	–	–
Saprophytic mycobacteria					
M. smegmatis-a	1+P	+	–	–	–
M. smegmatis-b	1+P	+	–	–	–
M. phlei-a	1+P	+	–	–	+
M. phlei-b	1+P	+	–	–	+
M. butyricum	1+P	+	–	–	+

P = pellicle formation. Numbers = the day growth was first observed.

On prolonged incubation, the thioglycollate may form flocculent or crystalline precipitates in the aerobic zone of the medium. This is due to concentration and may simulate growth. The crystalline material is probably mainly cystine, and the flocculent matter largely agar.[13] With some experience one can soon learn to distinguish this from growth. If there is doubt, smears and subcultures can be made and compared with control smears and subcultures prepared immediately after inoculating the medium and before incubating it. Thus far, the type of thioglycollate indicated has given the best results. It is also recommended that only fresh batches of medium be used.

SUMMARY

From these results it can be seen that fluid thioglycollate medium will not support the growth of any of the tubercle bacilli, but will support the growth of most of the atypical acid-fast bacilli, and all of the saprophytic mycobacteria. Growth of the atypical strains was slow and dysgonic, requiring from 11 to 39 days for initial development. On the other hand, growth of the saprophytes was rapid and eugonic, requiring only one day for initial development with characteristic pellicle formation.

On the basis of these and previous findings, it is concluded that fluid thioglycollate is useful as a selective medium for group differentiation of tubercle bacilli, atypical acid-fast bacilli, and saprophytic mycobacteria. For greater effectiveness, it is recommended that the thioglycollate test be used in conjunction with the peroxidase, neutral red, and serpentine cord tests.

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IDENTIFICATION OF ALKALOIDS AND RELATED COMPOUNDS BY PAPER CHROMATOGRAPHY AND ULTRAVIOLET SPECTROPHOTOMETRY

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SINCE the original work of Munier and Macheboeuf,[1-5] several reports concerning the identification of alkaloids and related basic drugs have appeared.[6-10] These procedures, employing paper chromatography, successfully separate and concentrate substances of forensic interest. The separated drugs can be located on the paper either by ultraviolet fluorescence or by reaction with spraying reagents. After location of these positive areas, the distance migrated from the point of origin is determined and then divided by the distance migrated by the solvent. This value (Rf) is consistent when identical conditions are employed. To further increase accuracy and overcome some of the variations encountered, it has been suggested that a reference standard be chromatographed simultaneously and Rf values be calculated in relation to the standard.[10] This would in effect correct for slight variations of temperature, pH, atmospheric solvent concentration, and other factors which may not be completely controlled.

Another analytic procedure which is useful for alkaloid identification is ultraviolet spectrophotometry.[11, 12] Most unsaturated organic compounds of forensic interest have unique absorption spectra, and this property has been used for direct identification. However, when dealing with biologic fluids and tissues, the presence of interfering substances requires preliminary concentration and purification prior to absorption spectrophotometry.

A combination of both of the above methods of identification has been used with successs at this laboratory.[9-11, 13] A combination of paper chromatography and ultraviolet absorption spectrophotometry was devised and reported for the identification of barbiturates.[14] The present report concerns the application of the two techniques for the identification of the basic alkaloids and antihistamines. A method of extraction of these materials from biologic fluids is also described.

MATERIALS AND METHODS

Biologic fluids and tissues were prepared for chromatography in the following manner: urine (10 to 30 ml.) or blood (25 ml.) is diluted to 50 ml. with distilled water. This sample

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is placed in a 250 ml. round-bottom boiling flask, fitted with ground-glass joint. Five milliliters of concentrated HCl is added and the mixture refluxed for 2 to 3 hours. The hydrolyzed sample is filtered and the filtrate is extracted with 15 to 25 ml. of ethyl ether. The ether layer is discarded and this procedure is repeated 5 times, discarding the ether (top) layer each time. The sample is then adjusted to pH 11 with 12 N NaOH. Extract the adjusted sample 5 times with 15 to 25 ml. portions of ether, pooling all of the ether extracts. This pooled ether extract contains all of the basic alkaloids. The sample is then readjusted to pH 9 with concentrated HCl. Extract this sample with 15 to 25 ml. of miscible solvent (chloroform: [95 per cent] ethanol, 4:1, v/v) and repeat this extraction 5 times, pooling all of the extracts. This pooled extract will contain all of the amphoteric alkaloids. Evaporate each of the pooled extracts to dryness. Transfer the residues to 10 ml. beakers with 95 per cent ethanol. Evaporate to dryness. This dried residue may be stored in a desiccator until ready for chromatography. When ready to chromatograph, add 0.1 to 0.2 ml. of absolute ethanol to the beaker, swirling to ensure solution. Ten μl (lambda) aliquots are spotted on the prepared buffered chromatography paper.

Chromatography.—Descending chromatography at 4 different pH's was employed for these studies. The buffers used were according to Goldbaum and Kazyak.[10] These buffers were as follows: pH 3.0 (790 ml. of 0.1 M citric acid diluted to 1 liter with 0.2 M sodium dibasic phosphate), pH 5.0 (480 ml. of 0.1 M citric acid diluted to 1 liter with 0.2 M sodium dibasic phosphate), pH 6.5 (680 ml. of M/15 potassium monobasic phosphate diluted to 1 liter with M/15 sodium dibasic phosphate), and pH 7.5 (164 ml. of M/15 potassium monobasic phosphate diluted to 1 liter with M/15 sodium dibasic phosphate).

Migrations were performed in a Chromatocab (Model A, RECO, Oakland, Calif.). The paper employed was Whatman No. 1, marked "for chromatography," 22½ by 18¼ inches in size. These large sheets were cut into strips 8 to 9 inches in width and 22½ inches in length. These strips were then completely wetted with the above-described buffers, each strip thus having a different pH. The strips were hung in a hood and allowed to dry for at least 4 hours. After allowing these strips to air dry, samples were spotted along a line approximately 3 inches from one end of the strip. The samples were spotted approximately 1 inch apart and drying was aided by the use of a hair dryer. The developing solvent employed was n-butanol saturated with the respective buffer solutions. The buffered papers were placed in the troughs and after 1 to 3 hours of equilibration, the appropriate buffer-saturated butanol was added (note: the tank atmosphere was presaturated by placing beakers of water-saturated butanol in the bottom of the tank 24 hours prior to chromatography). The system was allowed to migrate for 16 hours and for our purposes, overnight was found to be convenient. The paper strips were then removed, the solvent front marked, and the strips air dried. The dried strips were sprayed with iodoplatinate acid reagent,[10] which was made as follows: 45 ml. of 10 per cent potassium iodide, 5 ml. of 5 per cent platinum chloride, and 100 ml. of distilled water. The alkaloids and antihistamines appear as dark blue to black spots on a reddish-pink background. The point of maximum density was used to compute the Rf value.

Elution From Paper.—The method of Goldbaum and Kazyak[8] was modified as follows: the alkaloid-platinate spot was cut out and cut into minute pieces. These pieces were placed in a 50 ml. beaker and 3 ml. of Na_2SO_3-Na_2BO_4 solution (2 per cent Na_2SO_3 in saturated Na_2BO_4) was added. The material was mixed well to facilitate decomposition of the alkaloid-platinate complex. The supernatant was poured off into a separatory funnel. This procedure was repeated, combining the supernatants in the separatory funnel. Fifteen milliliters of miscible solvent (chloroform:ethanol) were added and the alkaloid extracted. This was repeated and the extracts combined. To the combined extracts, 5 ml. of 0.5 N HCl was added. The alkaloids were then extracted into the acid solution, the miscible solvent being discarded. This acid solution of the alkaloids was then ready for ultraviolet absorption spectrophotometry. For this purpose, a recording spectrophotometer (Beckman, Model DR or Cary, Model 11) was employed. A blank area of the paper strip was treated in a similar manner and was then used as the blank solution.

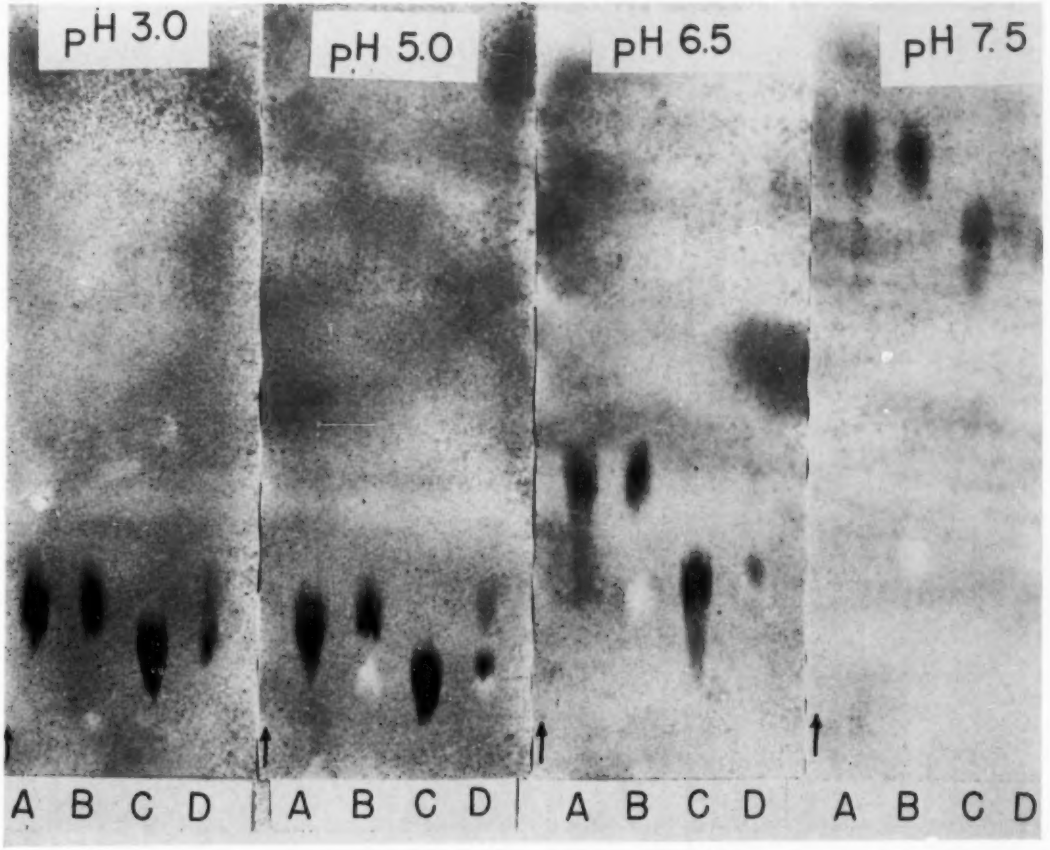

Fig. 1.—Results of chromatographic separation of urine extracts. Arrows indicate direction of flow of solvent. For each pH, A is the codeine standard, B is the codeine extracted from the unknown urine, C is the morphine standard, and D is the morphine extracted from the unknown urine. Note how the migration rates match.

RESULTS

The rates of migration of several alkaloids and antihistamines are listed in Table I. Four different pH levels were employed and a general increase in Rf

TABLE I. RATES OF MIGRATION (Rf) OF VARIOUS ALKALOIDS AND ANTIHISTAMINES

SUBSTANCE	pH			
	3.0	5.0	6.5	7.5
Dilaudid	0.09	0.08	0.16	0.67
Nicotine	0.07	0.19	0.71	0.84
Morphine	0.12	0.10	0.23	0.69
Codeine	0.19	0.18	0.35	0.77
Strychnine	0.21	0.20	0.29	0.67
Atropine	0.27	0.25	0.21	0.52
Neo-Antergan	0.28	0.58	0.60	0.85
Pyribenzamine	0.29	0.54	0.68	0.87
Cocaine	0.30	0.36	0.64	0.87
Heroin	0.31	0.37	0.58	0.83
Trimeton	0.31	0.54	0.56	0.87
Amphetamine	0.47	0.43	---	---
Sparine	0.52	0.52	0.59	0.84
Thorazine	0.56	0.59	0.67	0.86
Benadryl	0.55	0.52	---	---

value was obtained as the pH increased in alkalinity. However, at pH 3.0 and 5.0, most of the drugs of interest were sufficiently separated for tentative identification. At pH 5.0, all of the antihistamines tested exhibited Rf values greater than 0.50, a fact that made for easier identification of this class of compounds. At pH 7.5, most of the compounds migrated rapidly and differentiation was not as clear.

Since the typical sample to be analyzed at this laboratory was a biologic fluid or tissue, several known specimens were analyzed as a check of the accuracy of this procedure. Thus, the presence of any interfering substances which might tend to alter Rf values could be recognized. Such an example is illustrated in Fig. 1. Urine samples were obtained from a volunteer who had ingested 2 grains of codeine. The extraction procedure as outlined above was used and concentrates were chromatographed at 4 different pH levels. In each case, a standard solution was spotted and simultaneously chromatographed. Both codeine and morphine were recovered from the urine and, as illustrated, migrated at the same rates as the standards. It should be noted that the extraction procedure was specific in separating codeine and morphine, both being extracted at pH 9. On the other hand, only codeine was extracted at pH 11.

After calculation of the Rf values, these spots were eluted as described earlier in this report. Ultraviolet absorption spectra were obtained, recoveries ranging from 25 to 50 per cent. This was sufficient to produce readily identifiable spectra. An example of this was reported elsewhere.[13]

DISCUSSION

The chromatography of alkaloids and antihistamines indicated that, under proper conditions, satisfactory separation of these related compounds was obtained. Further, since a tentative identification could be made from Rf value alone, the presence of an alkaloid or antihistamine could be established rapidly.

In these cases, it is advisable to repeat the chromatography, including this time a sample or samples of known standards most closely approximating the unknown's migration rate. This serves a twofold purpose. First, it eliminates any slight changes in Rf caused by factors difficult to control (e.g., room temperature, atmospheric solvent concentration, variations in paper). Second, elution studies can then be performed from the same chromatogram on both unknown and standard, compensating for any alteration induced by the treatment. At the same time, an attempt at quantitation of the unknown is possible.

The use of a particular standard as a reference for migration calculations has been suggested and has definite merit. Thus, one can calculate not only Rf but also Rx, where x stands for the reference used. Although some have suggested the use of codeine for this purpose,[10] it has been our experience that the use of Pyribenzamine was better. Pyribenzamine migrates more rapidly than codeine and slight differences were more apparent. However, these calculations (R unknown/R Pyribenzamine) were not included in this paper. It is certainly advisable that each laboratory perform these calculations under their particular conditions.

The recoveries obtained by elution and destruction of the alkaloid-platinate complex were quite satisfactory for spectral identification of these substances. Spectral maxima and minima data have been recorded elsewhere[11] and were not included in this report. Like Rf data, it is necessary that each laboratory compile this information, using the instrumentation available to them. A recording spectrophotometer is an excellent instrument for these purposes.

Finally, the use of spectrophotometric screening has been employed as the initial step for unknown samples. The procedure outlined by Bradford and Brackett[11] has been used. Although it was rare that a positive identification could be made prior to purification by chromatography, several patterns of absorption were noticed, indicating certain families of drugs. For example, the presence of a barbiturate or barbiturates was indicated by an absorption peak at approximately 240 mμ in the "acid fraction." Alkaloids were indicated by an absorption peak at approximately 260 to 265 mμ in the "basic fraction." Thus, the screening procedure indicated what substances were present and what analytic procedures would be necessary for their identification. This combination of ultraviolet spectrophotometry and paper chromatography proved to be very successful in identifying some drugs of toxicologic interest.

SUMMARY

A procedure for the extraction and identification of several alkaloids and antihistamines is presented. Paper chromatography at 4 different pH levels separated most of the related drugs of forensic interest. Elution from the paper followed by ultraviolet absorption spectrophotometry was shown to be a further method of identification. These results are discussed.

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A TUBE TEST FOR Rh TYPING USING PAPAIN AND INCOMPLETE ANTI-D

Report of Two Separate Trials

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THE discovery by Morton and Pickles[1] that some proteolytic enzymes modified the red cell envelope and rendered treated cells agglutinable by certain incomplete anti-Rh sera resulted in the development of a simple but sensitive test for the detection of Rh antibodies. One disadvantage of such a two-stage method is that treatment of the cells and incubation of treated cells and serum under test must be done in two separate operations.

Löw[2] and Willert[3] independently reported that one-stage methods utilizing concurrent modification of the cells and incubation with the serum under test were practicable. Löw's papain method was originally designed for Rh typing, and provides a simple means of using readily available incomplete anti-Rh sera for testing saline suspensions of cells. Löw[4] reports that over 200,000 tests have now been done without any discrepancies other than those originally reported. Van Loghem[5] has also used this method on over 100,000 prenatal and donor specimens in parallel with saline anti-D; he feels that it is equivalent to the saline tube test, and slightly more sensitive than the albumin replacement method in detecting D variants.

Löw's papain method uses neutral cystein as an activator, and is an extremely sensitive reagent. But for routine Rh typing a simpler, stable, and rather less sensitive reagent would be preferable. The details of such a reagent developed in Sheffield by one of us (R. S.) are described below, together with the results of an extended trial in England and a later trial in Canada, in parallel with albumin-active and saline-active anti-D sera, respectively.

MATERIALS AND METHODS

Papain Solution (1 Per Cent).—

1. Mix 1 Gm. of papain with 100 ml. of M/15 phosphate buffer at pH 5.3. Shake well for 5 minutes, and leave overnight at 4° C. Papain 1: 350 obtained either from A. G. Merck, Darmstadt, Germany, or from British Drug Houses has proved satisfactory.

2. Filter the solution through Whatman No. 1 paper, then either centrifuge for 15 minutes or clarify through a Seitz C-5 pad.

3. The clear liquid is stored at – 20° C. in small (2 ml.) volumes until required.

Antisera.—

1. The Sheffield antisera were standardized by the methods described by Dunsford and Bowley[6]; the titer against CDe/cde (R,r) cells was no more than 1:8 by the albumin re-

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placement method. In every instance the specificity of the antisera was confirmed at the Medical Research Council Blood Group Reference Laboratory.

2. In Canada batches of incomplete anti-D prepared in the National Laboratory of the Canadian Red Cross Blood Transfusion Service were used. These antisera had a titer against albumin suspended CDe/cde (R_1r) cells of 1:64 when the tests were read macroscopically after 2 hours' incubation in a 37° C. water bath. The avidity of these antisera was such that good agglutination was present after 30 minutes' incubation. The specificity of all batches was tested by the indirect antiglobulin method as well as by that to be described before release to regional centers. The method of preparation and details of the techniques used have been given elsewhere.[7]

Cells.—Three to five per cent cell suspensions in 0.9 per cent saline were used throughout; the cells do not need to be washed in saline before use provided that the serum has been carefully removed. The source of the cells is described in the text.

Procedure.—

1. Thaw the papain solution immediately before use at no more than 37° C.; this step can be accomplished by holding the tube in the hand.

2. To one volume of anti-Rh serum, add one volume of 1 per cent papain and mix well. This mixture should be used within 30 minutes or else it will have to be discarded.

3. To one drop of a 3 to 5 per cent suspension of the patient's cells in 0.9 per cent saline, add one drop of the papain-serum mixture.

4. Shake to mix the contents, and incubate at 37° C. for 1¼ to 1½ hours.

5. The Sheffield tests were read microscopically, the Canadian tests macroscopically.

6. The cells used for the negative (absorption) controls should be group O Cde/cde (R_1r) and group A_1B or group A_1 and group B cde/cde.

RESULTS

Ten thousand prenatal specimens were typed by the papain serum method and with selected incomplete anti-D sera by the albumin replacement method[6]; the Sheffield results are shown in Table I.

TABLE I

GROUP	RESULTS WITH ANTI-D	NUMBER OF SAMPLES
I	Papain serum +	8,402
	Albumin replacement +	
II	Papain serum +	6
	Albumin replacement −	
III	Papain serum −	1
	Albumin replacement +	
IV	Papain serum −	1,584 (a)
	Albumin replacement −	7 (b)
	Total	10,000

The discrepancies in Groups II and III were all due to the presence of a D variant, as shown by a positive indirect antiglobulin test with incomplete anti-D serum, and could not be subscribed to the method. Seven samples containing a D variant were missed (Group IV, b) by both methods, which is not unexpected as the results are a function of the anti-D sera used and not of the methods.

In Canada, a pilot trial of Löw's papain was about to be made when it was learned[8] that the Sheffield technique of D-typing had been in routine use for some time. After preliminary tests confirmed the efficiency of the latter method, a trial was made in three centers of the Canadian Red Cross Blood Transfusion Service through the cooperation of Dr. T. D. Stout and Mrs. P. Lowery of

Vancouver, Dr. Lyonel Israels and Miss C. Anderson of Winnipeg, and Mrs. D. Watson of Toronto. By this method 3,148 blood bank specimens were tested in parallel with saline-active anti-D either of our own or of commercial manufacture. The results are given in Table II.

Of the 11 discrepancies in Group II, 9 were shown to possess a D variant, and one was not tested. One sample did not appear to possess a variant of D, because 3 batches of incomplete anti-D failed to sensitize the cells to an anti-globulin serum; however, this cannot be accepted as conclusive evidence that a variant of D is absent. Relatively more samples contain a D variant in Table II than in Table I, but this can be accounted for by the relative inability of saline-active anti-D to detect such variants when compared with albumin-active anti-D sera.

TABLE II

GROUP	RESULTS WITH ANTI-D		NUMBER OF SAMPLES
I	Papain serum	+	2,709
	Saline test	+	
II	Papain serum	+	11
	Saline test	−	
III	Papain serum	−	0
	Saline test	+	
IV	Papain serum	−	429
	Saline test	−	

DISCUSSION

Both the transfusion services concerned use precipitin (6.5 by 55 mm.) tubes and saline cell suspensions for routine tests on blood bank and prenatal specimens. If saline-active anti-Rh sera are not used, the available methods for use with incomplete antisera are limited, the most common being the albumin replacement method. The Canadian service did not consider the latter method suitable, and was loath to change to a slide test procedure. In both instances, a decision to investigate the use of papain in a one-stage technique was independently made. The results show that, whether compared with albumin-active or with saline-active anti-D sera, the papain serum method of concurrent treatment of the cells and incubation with the test reagent gives accurate results. Both services now use this method routinely for the initial D-typing of blood bank and prenatal specimens, as well as with other anti-Rh sera. The economy of the method is apparent, for the antiserum is diluted with an equal volume of papain before use; sera that are otherwise unsuitable have been found very satisfactory by this method.

The papain solution deteriorates at room temperature, and, of course, a mixture of this proteolytic enzyme and serum cannot be left for long before use. Tests have shown that, given a potent test reagent, there is no marked drop in titer or avidity within 1½ hours, but it is strongly recommended that papain serum mixtures be used within 30 minutes of preparation in order to provide an ample safety margin.

Another feature of papain serum mixtures is their ability to agglutinate coated cells; occasional false positive results may occur if the test cells have

cold incomplete antibodies adsorbed upon them or if the donor or patient has an acquired hemolytic anemia with autoantibodies. For the above reasons, it is not recommended that this method be used for Rh-typing patients prior to transfusions. With any sensitive method the use of adequate controls assumes greater importance than usual. Many incomplete anti-D sera originally contained anti-C which was removed by absorption during manufacture. The occasional reappearance of such an antibody, or of incomplete anti-A or anti-B, after a period of storage must be looked for by using suitable cells for the negative controls. All the tests described were made with antisera of our own manufacture; centers using slide test sera of commercial manufacture should make sure that the specificity, titer, and avidity of the antisera are adequate for this particular method before it is used routinely.

SUMMARY

A simple method of Rh typing using a one-stage papain serum method is described. No discrepancies were found in parallel tests with saline-active or with albumin-active sera in almost 14,000 tests.

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INFRARED ANALYSIS OF BLOOD SERUM, RED BLOOD CELLS, AND OTHER BODY FLUIDS

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INFRARED absorption spectroscopy has been used extensively to gather valuable information regarding the identity and structure of organic compounds.† It has proved to be, usually, the surest and most rapid method for the identification of single compounds or mixtures. These substances may be studied as gases, liquids, or solids. Solutions of substances are commonly used, but the solvent must be carefully selected for transparency to infrared radiation in the region to be studied. The two most commonly used solvents are carbon tetrachloride and carbon disulfide.

It is generally held that aqueous solutions can seldom be used because of the extremely strong absorption by liquid water throughout most of the infrared region. Hence, infrared spectroscopy has enjoyed only limited use as an instrument to study biologic systems.

In 1905, Coblentz[1] demonstrated that water could be used as a solvent for infrared absorption spectroscopy in some regions. In 1949, Gore, Barnes, and Petersen[2] obtained qualitative spectra of several amino acids and their salts in aqueous solutions at various pH levels. In 1956, Potts and Wright[3] demonstrated that quantitative infrared absorption spectroscopy can be carried out in water solution in a very thin absorption cell with barium fluoride windows. By using an improved double-beam infrared spectrometer featuring a larger optical system, excellent spectra were obtained in the 6.3 to 11 μ region. Interestingly, they published the first infrared spectrum of blood serum in this paper,‡ and suggested that their technique could be adapted for the direct examination of biologic fluids.

In 1958, the Medical Department and Spectroscopy Laboratory (Dow Chemical Company) examined a series of blood sera obtained from patients with one of the collagen diseases in an effort to ascertain whether infrared spectroscopy could detect differences between these and normal sera. As techniques were developed and improved, it became apparent that infrared analysis was sensitive to the serum protein changes occurring in the collagen diseases.

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†An excellent review of the theory and application of infrared absorption spectroscopy is to be found in Gilman's Organic Chemistry, Vol. III, Chapter II.

‡An infrared spectrum of *dried* blood serum was presented by J. T. Agnew, P. P. Lisan, and M. J. Boyd in 1952. J. Optic Soc. America, vol. 42, No. 11, 815-817.

It was then elected to examine normal blood cells, other body fluids, and blood sera from patients with various diseases in an effort to demonstrate the potential analytic prowess of this spectrographic method in the field of medical and biologic research. This paper is the summary of this endeavor.

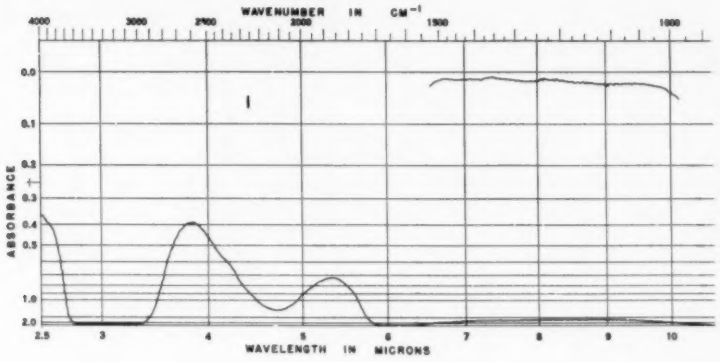

Fig. 1.—Distilled water.

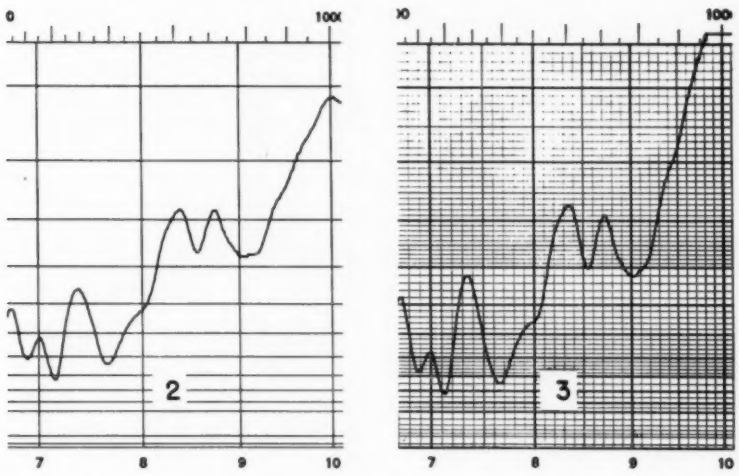

Fig. 2.—Specimen: whole blood. Reference cell: distilled water.
Fig. 3.—Specimen: adult red blood cells suspended in saline. Reference cell: 0.9 per cent saline.

MATERIALS AND METHODS

The following materials were investigated in their natural state, that is, no sample preparation was necessary: whole blood, human blood serum, rat blood serum, semen, urine, spinal fluid, ascitic fluid, pleural fluid, milk, saliva, and gastric juice. Saliva, spinal fluid, and one blood serum sample were concentrated about fivefold by vacuum drying at

room temperature. Red blood cells from oxalated blood were washed and resuspended in saline. Pathologic materials were obtained from patients with definitely established diagnoses.

Infrared Method.—The infrared spectrometer used in this work is the improved optical null instrument described by Herscher, Ruhl, and Wright.[4] The double-beam feature of the instrument makes it possible to scan 2 samples simultaneously, recording only the differences in their absorption spectra. These "differential scans" often indicate changes

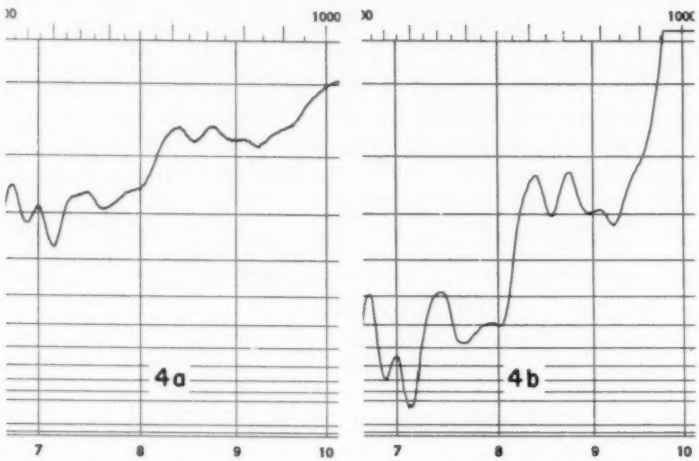

Fig. 4.—*a*, Specimen: human blood serum. Reference cell: distilled water. *b*, Specimen: human blood serum concentrated 5 times. Reference cell: distilled water.

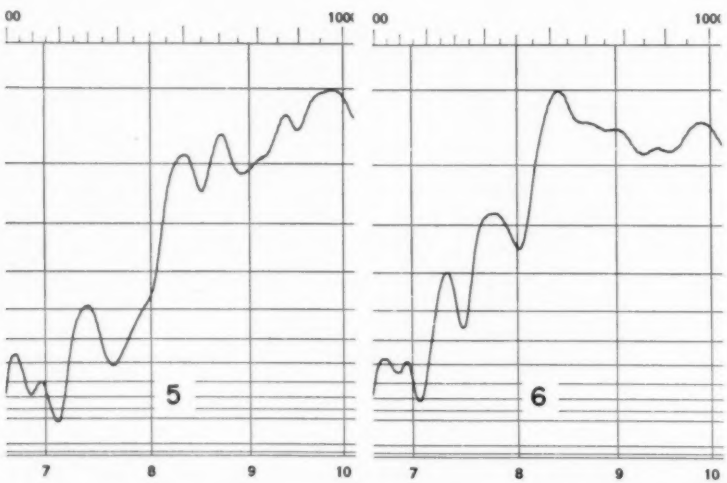

Fig. 5.—Specimen: serum albumin. Reference cell: saturated saline.
Fig. 6.—Specimen: serum gamma globulin. Reference cell: saturated saline.

which might otherwise go undetected. The large optical system of this spectrometer (150 mm. prism base) gives an adequate infrared signal at the detector for differential scans even in water solutions where the solvent alone may absorb more than 98 per cent of the incident radiation. This energy limitation has been a major obstacle to work on water systems in the past, but with the proper techniques and instrument settings the larger commercially available spectrometers should prove adequate for this type of work.[5, 6]

The instrument parameters are adjusted as follows: the slits are widened from the normal setting to give a twenty-five-fold increase of radiation at the detector, the scan time is increased to 12 minutes (about twice normal), and the optical null balance response time is also increased to twice normal. The manual gain is left at its normal setting, but the automatic gain feature of the instrument is used to compensate for the reduced infrared signal wherever the transmission in both beams is less than the maximum (2 per cent). In most of the scans the pen travel is normal, but in some cases the pen motion in the ordinate direction has been expanded about threefold to amplify small absorption differences. The most important change of those mentioned is the increase in slit width to maintain an adequate signal at the detector. The accompanying loss in resolution does not seriously affect the spectra because the absorption bands of biologic materials in water are fairly broad.

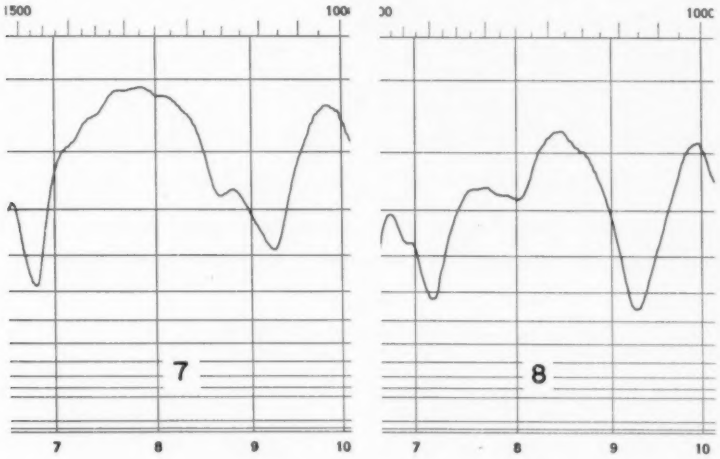

Fig. 7.—Specimen: human urine. Reference cell: distilled water.
Fig. 8.—Specimen: human semen. Reference cell: distilled water.

The sample cell, similar in design to that of Potts and Wright,[3] consists of 2 barium fluoride crystal windows held apart 0.06 mm. by a rectangular spacer of Saran rubber. Two drops of the specimen to be investigated are spread out between the barium fluoride plates, with the Saran rubber spacer serving to control the thickness and contain the sample. This assembly is clamped in a brass frame and placed in the instrument for scanning. No provision is made for filling the cell without disassembling it, but the cell thickness will reproduce within ±5 per cent on repeated assembling using the same spacer. It is advantageous to use a demountable cell of this type to facilitate cleaning of the sample chamber and to avoid the formation of bubbles when introducing the sample. In addition, by varying the pressure with which the plates are clamped together, it is possible to make small adjustments in the cell thickness necessary to match sample and reference absorptions on a differential scan.

When using water as the matching substance in the reference beam, its salinity should be matched to that of the biologic material, e.g., 0.9 per cent in the case of red blood cells suspended in saline. The presence of sodium chloride in aqueous solutions increases their transparency to infrared, particularly in the regions neighboring the cutoff wave lengths 6.3 and 11.0 μ.

The spectra presented are photographs of original tracings produced automatically by the spectrometer. They are reproduced at about ½ normal size.

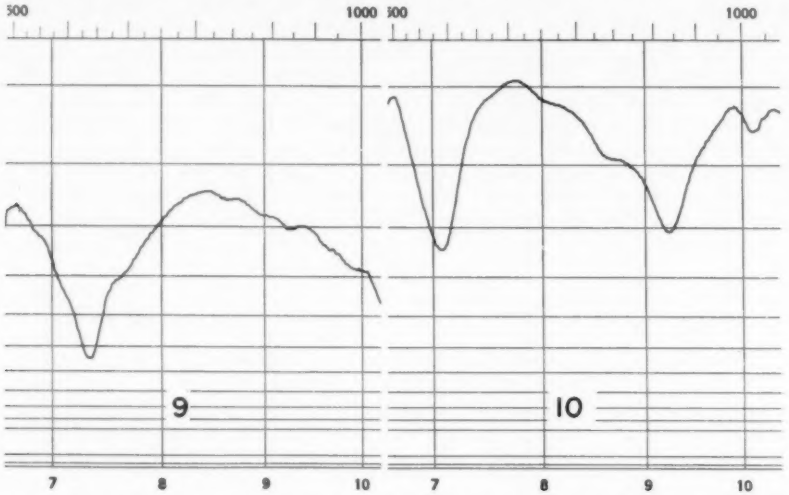

Fig. 9.—Specimen: cerebrospinal fluid concentrated 5 times. Reference cell: distilled water.

Fig. 10.—Specimen: human saliva concentrated 7 times. Reference cell: distilled water.

RESULTS

Fig. 1 represents an infrared spectrum of distilled water. Although adequate energy is available for obtaining infrared spectra between 3.5 and 5.8 μ, very few of the materials investigated have absorption bands in this region so it has not been included in the other charts. From 6.7 to 10 μ the transmission is low (2 per cent maximum); however, when distilled water is placed in both the sample and reference beams, both cells absorb equal amounts of the radiant energy and a balance is established which allows the relative transmittance to be raised to a suitable level (the higher curve in Fig. 1).

Figs. 2 through 11 represent spectra of various normal biologic materials in essentially their natural state. These spectra have been scanned with either distilled water or saline in the reference beam of the spectrometer. In many of the spectra an artifact appears at 7.5 μ which is due to the automatic insertion into the optical system of a filter for removal of scattered light. Two of the samples (4 and 9) have been concentrated to illustrate the increase in sensitivity which may be obtained by this means.

The infrared spectrum of whole blood is shown in Fig. 2. The absorption pattern of the red blood cells (see Fig. 3) dominates the spectrum, masking the bands of the serum and other cellular elements of blood.

Fig. 4, *a*, shows an infrared spectrum of human blood serum. A better spectrum is obtained when the serum is concentrated fivefold by evaporation (Fig. 4, *b*), but no new features are revealed. In these spectra the protein absorption bands predominate, masking the serum lipids and carbohydrates.

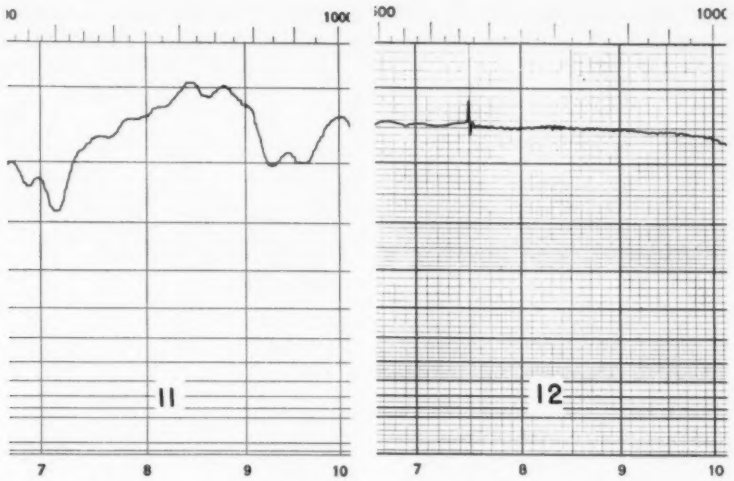

Fig. 11.—Specimen: human gastric juice. Reference cell: distilled water. (Note: spectrum obtained with pen expander.)
Fig. 12.—Specimen: normal human blood serum. Reference cell: normal human blood serum.

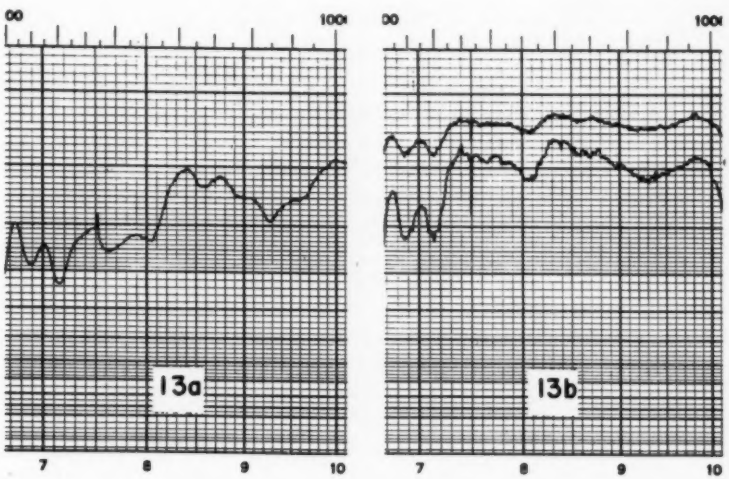

Fig. 13.—*a*, Specimen: blood serum from a patient with lupus erythematosus disseminatus. Reference cell: distilled water. *b*, Specimen: blood serum from a patient with lupus erythematosus disseminatus. Reference cell: normal serum.

Serum albumin absorption bands which contribute to the spectral pattern of serum are shown in Fig. 5. Fig. 6 shows the absorption pattern of serum gamma globulin. Gamma globulin has absorption bands at 7.5, 8.05, and 9.25 μ which are not present in the albumin pattern. The albumin fraction has absorption bands at 7.64, 8.5, 8.9, and 9.5 μ not present in the gamma globulin pattern. These absorption band differences arise from differences in the molecular structure of the two protein fractions and so afford easy differentiation between the two.

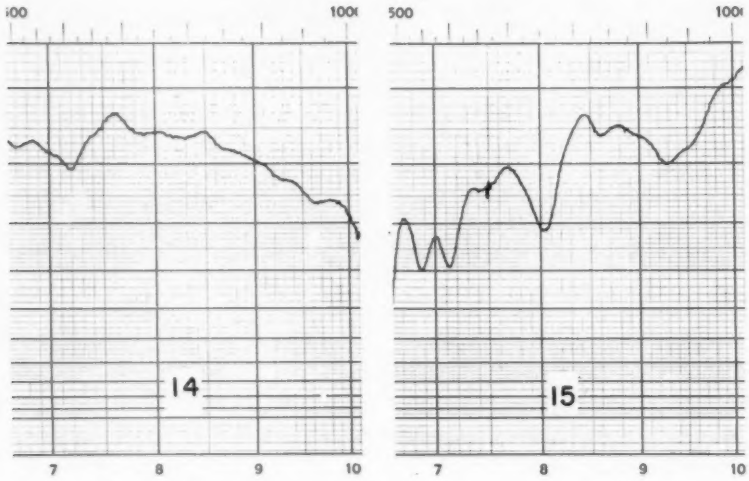

Fig. 14.—Specimen: blood serum from a patient with macroglobulinemia. Reference cell: normal serum.

Fig. 15.—Specimen: blood serum from a patient with multiple myeloma. Reference cell: normal serum.

The infrared spectrum of urine is shown in Fig. 7. Absorption bands at 6.8 and 8.7 μ are due to urea.

An infrared spectrum of human semen is shown in Fig. 8.

Unconcentrated cerebrospinal fluid has only one broad absorption band at 7.33 μ. When concentrated 5 times by evaporation, several additional bands become evident, as shown in Fig. 9.

Both human saliva and gastric juice have interesting spectra, pictured in Figs. 10 and 11, respectively.

Fig. 12 demonstrates the flat base line obtained when 2 identical blood sera are scanned differentially to cancel their common absorption bands. The small deflections are due in part to a slight mismatch of cell thicknesses and in part to random noise in the instrument.

Figs. 13 through 24 are infrared spectra of blood serum from patients with definitely established diagnoses. These were obtained with either water or normal blood serum in the reference beam. In the latter case, the absorption bands noted represent differences in the chemical make-up between the two sera.

Fig. 13, *a*, is the infrared spectrum of blood serum from a 21-year-old woman with the established diagnosis of lupus erythematosus disseminatus. The bands at 8.05 and 9.27 μ are deeper than those of the normal blood serum. Fig. 13, *b*, illustrating the differential run between this serum and the control serum, confirms that there are absorption bands at 8.05 and 9.27 μ which do not appear in normal serum. Additional bands at 6.85, 7.15, and 8.55 μ are also revealed.

An infrared spectrum of blood serum from a patient with macroglobulinemia, scanned with distilled water in the reference beam, was noted to

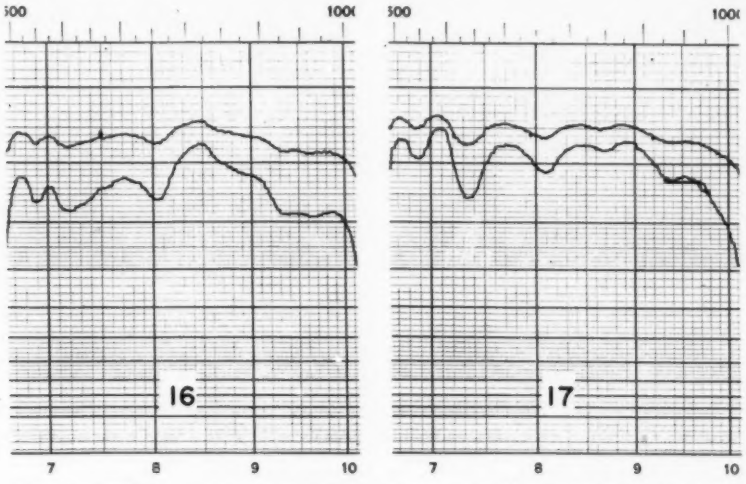

Fig. 16.—Specimen: blood serum from a patient with aplastic anemia. Reference cell: normal serum. (Note: Lower tracing obtained with pen expander.)

Fig. 17.—Specimen: blood serum from a patient with panhypopituitarism. Reference cell: normal serum. (Note: Lower tracing obtained with pen expander.)

possess a different configuration than the normal blood serum. The absorption band at 7.13 μ was deeper and broader than seen in the normal serum, the normally present band at 7.6 μ was absent altogether, and a new band appeared at 9.6 μ. The differential scan, as shown in Fig. 14, reveals the presence of a strong band at 7.2 μ, shows the absence of the 7.6 and 8.5 μ bands by an upward deflection of the pen at these points, and confirms the new band at 9.6 μ.

Fig. 15 represents the differential scan of blood serum from a patient with multiple myeloma run with normal serum in the reference beam. The absorption bands at 6.88 and 7.14 μ are deeper than those of the normal serum. In addition, a strong negative deflection at 7.71 μ, a very strong band at 8.05 μ, and a band at 8.6 μ in place of the 8.55 μ band are noted. Two additional blood serum samples from patients with multiple myeloma have been scanned differentially, and it is encouraging that the differences found in the first pathologic serum are reproduced in these two. Interestingly, several differences of lesser magnitude were observed between the multiple myeloma sera in the 8 to 10 μ region. An explanation of these spectral differences awaits further study.

A differential scan of blood serum from a patient with aplastic anemia run against normal serum is shown in Fig. 16. Broad deep bands are present at 6.9, 7.2, and 8.05 μ. Two small bands are present at 9.35 and 9.65 μ. The spectrum of the serum from this patient with aplastic anemia obtained with

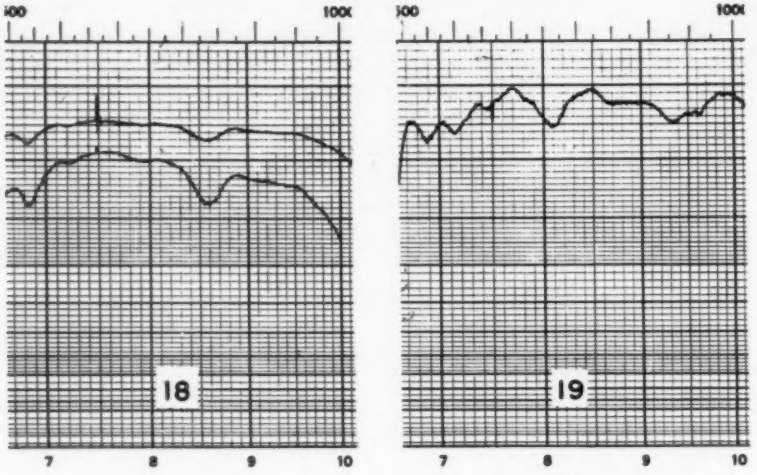

Fig. 18.—Specimen: blood serum from a patient with monomyelocytic leukemia. Reference cell: normal serum. (Note: Lower tracing obtained with pen expander.)

Fig. 19.—Specimen: blood serum from a patient with cystic fibrosis of the pancreas. Reference cell: normal serum.

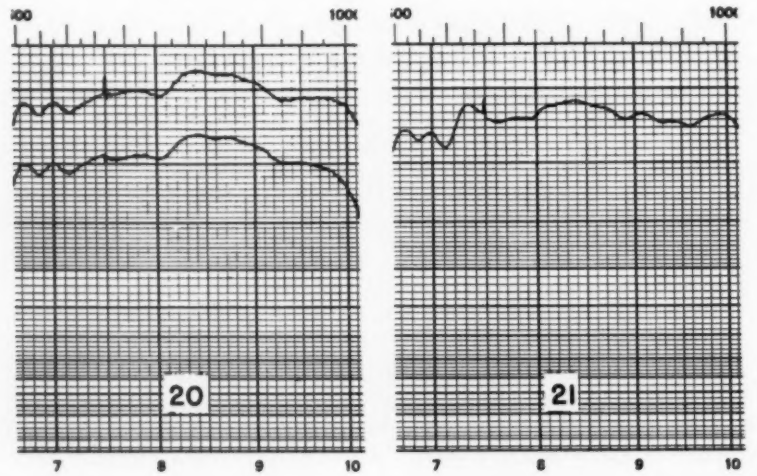

Fig. 20.—Specimen: blood serum from a patient with salivary gland carcinoma (2 scans). Reference cell: normal serum.

Fig. 21.—Specimen: blood serum from a patient with an acute myocardial infarction. Reference cell: normal serum.

distilled water in the reference beam was essentially the same as that of normal serum, except that the band at 8.05 μ was more prominent in the former.

A spectrum of blood serum from a patient with panhypopituitarism closely resembles the control serum spectrum. Fig. 17, showing the differential scan between the two sera, reveals the previously obscured absorption bands at 7.35, 8.1, 8.7, and 9.4 μ.

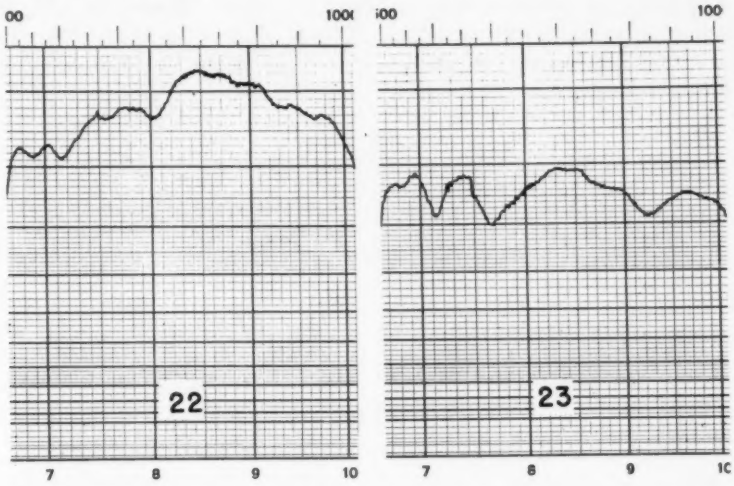

Fig. 22.—Specimen: blood serum from a rat exposed to carbon tetrachloride. Reference cell: normal rat serum.
Fig. 23.—Specimen: fetal red blood cells. Reference cell: adult red blood cells.

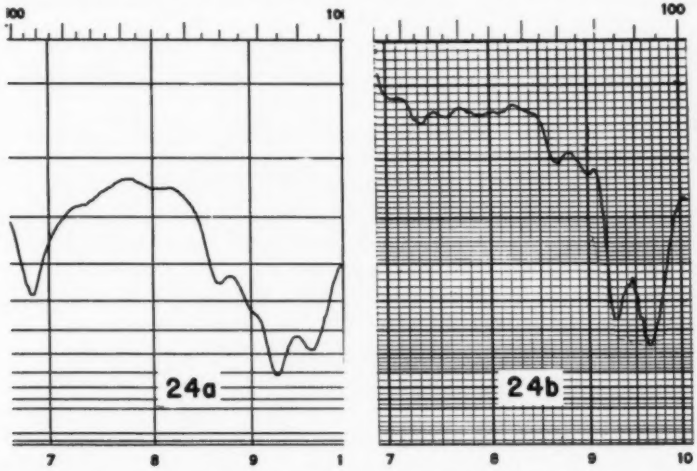

Fig. 24.—*a*, Specimen: human urine with 4 plus sugar level. Reference cell: distilled water *b*, Specimen: human urine with 4 plus sugar level. Reference cell: normal urine.

Figs. 18 through 20 represent differential spectra of various pathologic sera versus normal sera. These are presented to show that these disease states feature sera with infrared detectable differences.

Fig. 21 represents the differential spectrum of serum from a patient with an acute posterior myocardial infarction and was obtained on the third day following the infarction. The serum glutamic-oxalacetic transaminase (SGOT) was sharply elevated.

A differential spectrum between sera from a rat exposed to CCl_4 and a control rat is shown in Fig. 22. The rat was exposed to CCl_4 7,000 ppm by inhalation for 2 hours with the serum obtained immediately following exposure. The SGOT was elevated.

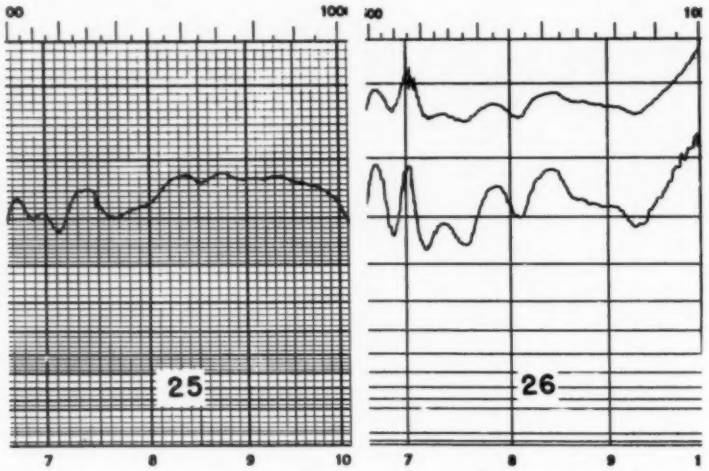

Fig. 25.—Specimen: serum albumin from a patient with Hodgkin's disease. Reference cell: distilled water.

Fig. 26.—Specimen: serum gamma globulin from a patient with multiple myeloma. Reference cell: distilled water.

The infrared spectra of red blood cells from a series of specimens of cord blood were essentially the same as the spectrum of the adult red blood cells in Fig. 3. Fig. 23 reveals a representative differential run between one specimen of fetal cells and adult red cells. An absorption band is observed in the fetal cells at 9.2 μ which is not present in the adult cells. Minor differences between the fetal cells were found in the 8 to 9 μ region.

Fig. 24, *a*, shows a spectrum of a urine featuring a 4 plus sugar level obtained from a badly burned child. The absorption pattern produced when this urine is scanned differentially (Fig. 24, *b*), against normal urine is the same as that of the glucose solution the patient was receiving intravenously.

Examples of infrared spectra of serum protein fractions obtained by curtain electrophoresis are presented in Figs. 25 and 26. A spectrum of serum albumin from a patient with Hodgkin's disease is shown in Fig. 25. Fig. 26 shows the spectrum of gamma globulin obtained from a patient with multiple myeloma.

Although these spectra are essentially the same as those of normal albumin and gamma globulin fractions (Figs. 5 and 6, respectively), small differences between normal and pathologic serum fractions might remain undetected unless the differential method was employed.

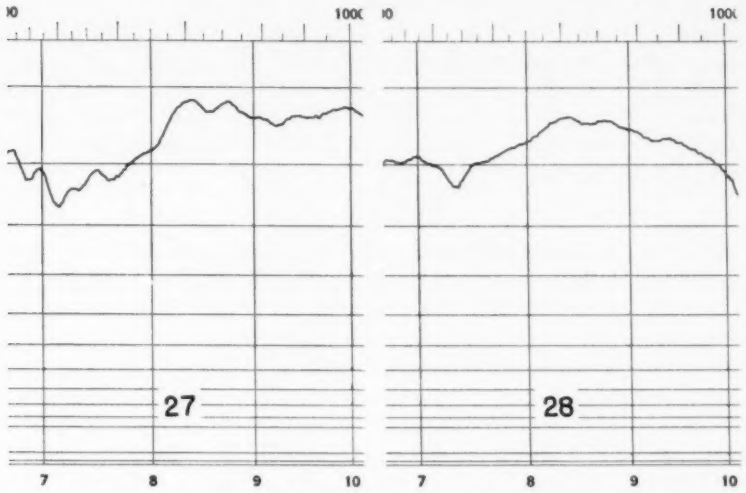

Fig. 27.—Specimen: pleural fluid from a patient with chronic lymphocytic leukemia. Reference cell: distilled water.

Fig. 28.—Specimen: ascitic fluid from a patient with carcinomatosis. Reference cell: distilled water.

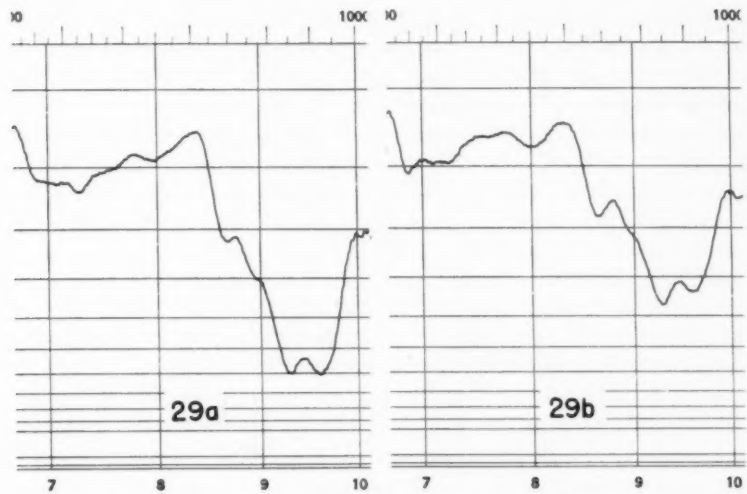

Fig. 29.—*a*, Specimen: human milk diluted to 50 per cent with distilled water. Reference cell: distilled water. *b*, Specimen: cow's milk diluted to 50 per cent with distilled water. Reference cell: distilled water.

Fig. 27 shows the infrared spectrum of pleural fluid obtained from a patient with chronic lymphocytic leukemia. It appears to be very similar to the spectrum of blood serum from the same patient.

The infrared spectrum of ascitic fluid from a patient with carcinomatosis is shown in Fig. 28. Its similarity to spinal fluid is striking.

Human breast milk (Fig. 29, *a*) gives a dramatically different infrared spectrum from the fluids previously examined. The high lactose content (7.5 per cent), high fat content (3.5 per cent), and relatively low protein content (1.25 per cent) account for these differences. A spectrum of cow's milk (Fig. 29, *b*) is presented for comparison. Both have been diluted with an equal volume of distilled water.

DISCUSSION

Now that water solutions are no longer an insurmountable barrier, infrared absorption spectroscopy has much to offer in the analysis of biological systems rich in water content for a variety of reasons. (1) Each organic compound in a biologic system has its own specific infrared absorption spectrum. (2) Inorganic molecules and ions which contain covalent bonds possess a characteristic infrared absorption spectrum. (3) In a mixture of compounds the spectrum of the mixture is the sum of the spectra of the individual components, and the absorbance due to any component is proportional to its concentration. (4) Two complex chemical systems, each of which contains many of the same but also different compounds, may be scanned differentially, allowing only the chemical differences to be recorded.

Although blood serum is a very complex chemical system, it is readily obtained and seemed a promising field for study. It was necessary first to ascertain whether a reliable spectrum of unconcentrated serum could be obtained and, if so, whether infrared differential techniques would be sensitive enough to detect differences between sera from patients in health and in disease. Using the methods outlined above, good spectra of 60 normal sera were obtained. These were found to be essentially the same as the control sera. At the same time spectra of a number of pathologic sera were obtained, most of which showed significant deviations from the normal pattern. The possibility of using these deviations without interpretation for diagnostic purposes was at once suggested. This would, of course, require an infrared analysis of sera from a statistically significant number of patients with a single disease. As such a series of samples was not readily available an investigation of those absorption differences which had been observed was begun.

As a first step in identifying the absorption bands in these sera, the albumin and gamma globulin fractions from normal serum were obtained and their infrared spectra determined. These reference spectra explained many of the bands found in normal serum and, in addition, gave information about some of the substances producing the differences observed in the pathologic samples. For example, the band at 8.05 μ observed in many of the pathologic sera (see Figs.

13, 15, 17, 18, and 19) is certainly due, at least in part, to gamma globulin levels above those in normal serum. Variations in the absorptions at 7.64 and 8.50 μ are very probably caused by changes in the albumin level. These two bands are reduced in intensity in Fig. 14 (slight upward deflection of the pen) indicating albumin levels below those in normal serum. These interpretations must, of course, be regarded as tentative until the spectra of a complete set of protein fractions from normal serum are obtained.

Fractionation procedures may also be performed on pathologic sera and, in fact, will probably be required to determine which serum constituents represent the abnormality. Moreover, many clues as to the molecular structure of the chemical entities producing the "abnormal" absorptions may be gained from expert interpretation of the spectra. The possibility of using differential scans of the protein fractions (abnormal versus control) as a diagnostic tool is also being investigated. It is hoped, however, that after the initial assignment of absorptions has been made that most diagnostic work can be performed on unfractionated samples in order to realize fully the rapidity of the method.

The infrared study of the intact living cell is also very promising. Because the red blood cell strongly absorbs infrared energy, it produces an excellent infrared spectrum. In our small series a consistent difference was found between the adult red blood cell and the fetal red blood cell. In addition, small differences in the structure of the fetal red cells themselves were noted. This suggests that structural differences in the red blood cells in the hemoglobinopathies should be investigated by this technique, and the method adapted to the study of many cellular elements in blood and body fluids. Investigation of tissue culture cells and the changes they produce in their nutrient media opens even another realm for infrared research. Again, fractionation procedures will facilitate the identification of the chemical constituents responsible for differences observed in the cellular elements.

The infrared spectra of other body fluids have been presented to demonstrate that virtually any material of this type may be examined by infrared. The procedures used in examining blood sera may be applied equally well to these other materials to detect the metabolites found in various disease states.

SUMMARY

A rapid method is described for the infrared examination of blood serum, living cells, and biologic fluids which requires but 2 drops of sample. Spectra of red blood cells and a variety of normal and pathologic biologic fluids in their natural state are presented, along with spectra showing significant differences between these normal and pathologic fluids. The possibility of using these spectra for diagnosis, qualitative analysis, and molecular structure research in the medical field is discussed.

We wish to acknowledge the splendid cooperation on the part of the Department of Internal Medicine, University of Michigan, and the Staff of the Midland Hospital who supplied us with most of the pathologic specimens.

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AN IMPROVED RAPID METHOD FOR FREE AND CONJUGATED 17-HYDROXYCORTICOSTEROIDS IN PLASMA

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MOST of the methods now in use for the determination of 17-hydroxycorticosteroids (17-OHCS) are concerned with the estimation of free (unconjugated) steroids in plasma[1-4] or their conjugates in urine.[5, 6] Although methods for the estimation of conjugated 17-OHCS in plasma have been described,[7-9] they have not been adopted for wide use in clinical investigation because they are too complicated for work in a routine clinical laboratory.

This paper deals with the development of a simple but sensitive method for free and conjugated 17-OHCS, both being estimated on the same sample of plasma. The method obviates some of the complicated procedures of the methods hitherto available, such as column chromatography, evaporation to dryness of extracts in high boiling solvents, and the use of microcuvettes, and can be easily employed in routine clinical investigation.

MATERIALS AND METHODS

Reagents.—

1. Chloroform: redistilled freshly from anhydrous potassium carbonate.
2. n-Butanol (analytical reagent grade).
3. Anhydrous sodium carbonate, powder (analytical reagent grade).
4. Methyl alcohol: distilled twice from 2:4-dinitrophenylhydrazine.
5. Carbon tetrachloride (analytical reagent grade).
6. Acetate buffer (2 M, pH 4.5).
7. Hexane (boiling range 67° to 70° C., reagent grade).
8. Ethyl alcohol: distilled twice from 2:4-dinitrophenylhydrazine.
9. Barium reagent: 40 per cent w/v barium acetate (monohydrate) in water.
10. Sulfuric acid (25 per cent v/v).
11. Anhydrous ammonium sulfate (analytical reagent grade).
12. Sulfuric acid-blank reagent: 62 per cent v/v H_2SO_4 (analytical reagent grade).
13. Phenylhydrazine-sulfuric acid reagent: 65 mg. of phenylhydrazine hydrochloride (twice recrystallized from ethyl alcohol) redissolved in 100 ml. of 62 per cent sulfuric acid.
14. Beef liver β-glucuronidase (Ketodase): 5,000 units per milliliter.

Plasma Free 17-Hydroxycorticosteroids.—

Technique: Ten milliliters of heparinized plasma (0.2 ml. of heparin = 100 units per 20 ml. of blood), separated immediately after withdrawal of blood, is extracted with 50 ml. of chloroform in an 80 to 90 ml. glass-stoppered test tube by vigorous shaking for 30 seconds.

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The tube is centrifuged for 15 minutes at 2,500 r.p.m. and the extract is fully separated off by means of a pressure operated liquid separator (Fig. 1) and is transferred to another glass-stoppered test tube. Two milliliters of n-butanol are added, the tube is stoppered and the contents are mixed by inversion. The extract is refrigerated for 30 minutes (0° to 4° C.), then 0.6 Gm. of anhydrous Na_2CO_3 is added and the tube is shaken vigorously for 20 seconds. After centrifugation for 10 minutes at 2,500 r.p.m. the extract is decanted directly to a 50 ml. measuring cylinder. The whole extract is divided into two equal parts using measuring cylinders and transferred to two 40 ml. glass-stoppered test tubes. To one, 2 ml. of reagent blank is added, to the other 2 ml. of phenylhydrazine-sulfuric acid reagent from burettes. The tubes are stoppered and shaken intensively for 15 seconds. After standing for 30 minutes followed by a short centrifugation the chloroform layer is carefully removed by means of a 7 inch blunt needle connected to an aspirator (water pump) controlled with a two-way glass tap. The remaining phase is then incubated by standing in the dark at room temperature for 18 hours or by heating in a water bath at 60° C. for 40 minutes. The developed color is then stable for at least 20 hours.

Standards of hydrocortisone are run in parallel: duplicates of 5 and 10 μg of hydrocortisone in 1 ml. of n-butanol are combined each with 25 ml. of redistilled chloroform in 40 ml. of glass-stoppered tubes. Then 2 ml. of sulfuric acid-blank reagent and phenylhydrazine-sulfuric acid reagent, respectively, are added and the procedure is followed as described above.

Blanks are obtained by combining 1 ml. portions of n-butanol each with 25 ml. of chloroform and subsequent shaking with 2 ml. of sulfuric acid blank reagent and phenylhydrazine-sulfuric acid reagent, respectively, then proceeding as above.

After simultaneous incubation of the blanks, standards, and the plasma extracts, optical densities are read in a Unicam (SP 500 or SP 600) spectrophotometer at a wave length of 410 mμ in standard 20 mm. cuvettes. The use of a diaphragm reducing the length of the hole in the cell-holder is necessary in view of the small volume of fluid. (If the amount of free 17-OHCS is expected to be above 20 μg per 100 ml. of plasma, standard 10 mm. cuvettes may be used without diaphragm.) Colors with sulfuric acid alone are read against sulfuric acid blank, colors with phenylhydrazine against phenylhydrazine-sulfuric acid blank, the reading being made first for sulfuric acid colors and then for phenylhydrazine.

Plasma Conjugated 17-Hydroxycorticosteroids.—

Technique: To the residue of plasma after extraction by chloroform (still in the same tube) 20 ml. of redistilled methyl alcohol is added from a burette, the tube being simultaneously agitated. It is then stoppered and the contents are thoroughly mixed by inversion. Forty milliliters of carbon tetrachloride are added and the mixture is vigorously shaken for 45 seconds. After standing for 20 minutes the tube is centrifuged for 15 minutes at 2,500 r.p.m. The alcoholic layer is then easily decanted into another clean 80 ml. glass-stoppered test tube. It is subsequently acidified to pH 4.5 to 5.0 (indicator paper) by addition of acetate buffer (usually 25 drops are sufficient) and washed twice with 40 ml. of hexane by shaking each time for 30 seconds and centrifuging for 8 minutes at 2,500 r.p.m. The hexane layer is removed by means of a capillary pipette connected to an aspirator (water pump). The alcoholic layer is then decanted* into another clean 40 to 50 ml. glass-stoppered test tube; 10 ml. of redistilled ethanol and 0.2 ml. (8 drops) of barium reagent are subsequently added, the contents are mixed by inversion, and the tube is placed in a refrigerator for 30 minutes. It is then centrifuged for 10 minutes at 2,500 r.p.m. and the supernatant is quantitatively transferred (decanted) into a 150 ml. round-bottomed flask and evaporated to dryness at a temperature of 45° C. (water bath) under reduced pressure (with air). Care should be taken that the residue is dried completely (see experimental part), this being achieved by repeated additions of methanol to the flask during the evaporation procedure. The dry residue is redissolved in 4 ml. of distilled water, acidified to pH 1

*The white sediment remaining in the tube does not contain any 17-OHCS and should some of it be transferred to the flask during the decantation procedure it will not appreciably increase the color with the sulfuric acid reagent. The investigation into the nature of the sediment, which has been identified as α_1-glycoprotein, is to be published separately.

by dropwise addition of 25 per cent sulfuric acid, allowed to stand for 15 minutes, and saturated with 3.5 to 4.0 Gm. of anhydrous $(NH_4)_2SO_4$. Four milliliters of n-butanol and 40 ml. of chloroform are subsequently added and the flask is shaken vigorously for 3 minutes. The contents are then fully transferred to a 50 to 60 ml. test tube, centrifuged for 10 minutes at 2,500 r.p.m., and the chloroform-butanol layer is carefully separated off by means of a pressure operated liquid separator as described before. The extract is then filtered through Whatman No. 1 filter paper (diameter not larger than 9 cm.) into a 50 ml. measuring cylinder and divided into two equal portions, which are transferred to two 40 ml. glass-stoppered test tubes. To each of them 10 ml. of redistilled chloroform* is added, followed by 4 ml. of sulfuric acid-blank reagent and phenylhydrazine-sulfuric acid reagent, respectively; the tubes are stoppered and shaken vigorously for 15 seconds. Then the procedure is followed as for free steroids, the incubation time being 45 minutes if heating at 60° C. in a water bath or 34 hours if at room temperature (in the dark). The cuvettes are used with 40 mm. long light path (using diaphragm attachment) or 20 mm. if the expected concentration of 17-OHCS is above 20 μg per 100 ml. of plasma.

Fig. 1.—A pressure operated liquid separator (reg. patent). The thick-walled glass-stoppered test tube (A) in which the extraction took place is fixed after centrifugation in the stand (H) on the eye-level of the operator. The head (B) is then firmly inserted, the end of the stem (D) almost touching the bottom of the tube. The blow-ball (F) is connected to the side tube (E) and the air pressure above the liquid is gently increased, keeping the tap (C) closed, until the rubber reservoir (G) is filled adequately with air. Then, by gradual opening of the tap (C), the bottom layer is removed into the head (B), forced there by the increased air pressure. A quick closure of the tap assures discontinuation of the removal at the required moment. The head is then disconnected and the separated layer is released into a clean container. (Minute contaminations from the upper layer which may enter the stem at the time of its insertion are retained on the wall of the head during subsequent release of the separated fluid.) The extraction, followed by removal of the extract, may be repeated on the same residue as many times as necessary.

*The addition of chloroform is not essential, but it results in more favorable distribution of some fatty material between the chloroform and butanol-sulfuric acid layers, thus further decreasing the intensity of the background color produced with sulfuric acid.

Standards and blanks are accordingly obtained by mixing 2 ml. portions of hydrocortisone in n-butanol and pure n-butanol, respectively, with 30 ml. of chloroform and subsequent extraction by 4 ml. of reagents (sulfuric acid-blank and phenylhydrazine-sulfuric acid, respectively).

Separate Determination of 17-Hydroxycorticosteroid Glucuronides.—The plasma specimen, previously extracted with chloroform, is deproteinized with methyl alcohol and distributed between the aqueous alcohol and carbon tetrachloride and hexane, respectively, the procedure here being exactly the same as the one described for the corresponding part of the method for total conjugated 17-OHCS. The purification step with the barium reagent, before the evaporation to dryness, is omitted. The dry residue in the 150 ml. round-bottomed flask is then redissolved in 10 ml. of distilled water, acidified to pH 4.5 (indicator paper) with acetate buffer, and hydrolyzed with 2 ml. of β-glucuronidase (10,000 units) at a temperature of 37° C. (incubator) for 46 hours. The hydrolyzed material is then allowed to cool to room temperature and is extracted with 5 volumes of redistilled chloroform. After separation of the extract 2 ml. of n-butanol are added and the procedure followed is exactly the same as that described for free 17-OHCS.

EXPERIMENTAL PROCEDURES

The Free (Chloroform Soluble) 17-Hydroxycorticosteroids.—Experiments in which anhydrous sodium carbonate was used for the purification of chloroform extracts showed that considerable loss of 17-OHCS occurred during the purification. No 17-OHCS could be recovered from the carbonate; it was concluded that they were destroyed by it. As this destruction did not occur when n-butanol extracts were purified with carbonate, the possibility of preventing the loss by adding pure n-butanol to the chloroform extracts, prior to the purification procedure, was studied. It was seen that as little as 2 ml. of n-butanol per 50 ml. of the chloroform extract fully protected the free 17-OHCS from destruction by carbonate.

This method of purification has several advantages: The recovery of free hydrocortisone is complete, while the use of liquid reagents—sodium hydroxide, sodium bicarbonate, sodium chloride— causes some loss of steroids in chloroform extract. The method is technically simpler than those using liquid reagents. The purification is satisfactory as negligible sulfuric acid blank values result. Furthermore, the addition of n-butanol to the chloroform extracts assures high stability of the extracts. Their storage up to 6 days in a refrigerator or at room temperature did not cause any appreciable loss in the steroid concentration.

Methyl, ethyl, amyl, and caprylic alcohols were also tested as to their abilities to protect the free 17-OHCS from destruction by anhydrous carbonate; n-butanol was found to have the most advantages.

The phenylhydrazine reagent does not include ethyl alcohol as in the Silber-Porter method[1]; it re-extracts the 17-OHCS together with the alcoholic phase from the chloroform-butanol extract, what presumably accounts for the better recoveries at this stage, than those at the corresponding stage of the Silber-Porter method (Table I). The phenylhydrazine-sulfuric acid reagent is stable for 10 days if kept in a refrigerator.

Experiments designed to establish the optimal chloroform-butanol and butanol-acid ratios fixed the respective figures for 25:1 and 1:2. Under these circumstances the maximum decrease in the color produced with sulfuric acid alone and full recovery of 17-OHCS is achieved.

TABLE I. RECOVERIES OF FREE 17-OHCS AT THE RE-EXTRACTION STAGE OF THE CHLOROFORM EXTRACT WITH THE SULFURIC ACID REAGENT. COMPARISON OF THE SILBER-PORTER METHOD WITH THE METHOD DESCRIBED IN THIS PAPER

MATERIAL RE-EXTRACTED	METHOD OF RE-EXTRACTION	FREE 17-OHCS		
		ADDED	RECOVERED	
		μg/100 ML.	μg/100 ML.	PER CENT
Standard hydrocortisone solution	Silber-Porter	400	352	88
Standard hydrocortisone solution	Butanol-steroid phase re-extraction	400	400	100
Urinary extract	Silber-Porter	90	77	86
Urinary extract	Butanol-steroid phase re-extraction	90	90	100
Plasma extract	Silber-Porter	40	30	75
Plasma extract	Butanol-steroid phase re-extraction	40	38	95

The Conjugated 17-Hydroxycorticosteroids.—It was assumed that the butanol-chloroform mixture could be used for the extraction of conjugated steroids from plasma, as it proved to be satisfactory when applied to urine.[10] Although emulsions formed by this mixture with plasma proteins are easily breakable, several difficulties were encountered, which had to be overcome by the introduction of various preparatory procedures, prior to the actual extraction. The acidification of plasma to pH 1, indispensable to obtain full extraction of the conjugates, caused rapid breakdown of some of the proteins, liberating certain lipids and other nonspecific chromogenic material extractable by the butanol-chloroform mixture. The saturation of acidified plasma with ammonium sulfate, also necessary for the complete extraction of the conjugated 17-OHCS into the butanol-chloroform mixture, further increased the amount of extracted interfering chromogens.

Methods to remove this material were then investigated. Duplicates of plasma were deproteinized with ethyl and methyl alcohols, respectively. Both alcohols were used in such quantities as to give strengths of about 70 per cent when mixed with the water content of plasma. The proteins were then discarded and the aloholic phase washed with hexane. It was noticed that a lower pH (4.5 to 5.0) was more favorable for the desirable partition of lipoid material. The acidification with acetate buffer proved to be superior to that by the acetic acid and it was consequently adopted, especially in view of the protective properties of the buffer in respect to steroids during the subsequent evaporation to dryness.

Considerable differences were noted in the mode of precipitation by ethyl and methyl alcohols. Ethyl alcohol (70 per cent) caused more denaturation of certain proteins (lipoproteins and presumably mucoproteins) than methyl alcohol. This resulted in greater amounts of interfering chromogenic material in the ethanolic extracts. Furthermore, 70 per cent ethanol retained more lipids during the subsequent partition with hexane than 70 per cent methanol. Methyl alcohol was adopted, therefore, for the procedure.

Various fat solvents (hexane, benzene, carbon tetrachloride, and ether) were tested before carbon tetrachloride was finally adopted for the method. It was added to plasma immediately after the deproteinization with methyl alcohol but before centrifugation and discarding of precipitated proteins. The separation of proteins is then quicker (due to greater differences in the specific gravity of the respective components) and the protein layer is more compact. This also decreases the possibility of some loss of steroids in the liquid content of the protein layer. The alcoholic phase is easily decanted after centrifugation, as the protein layer forms a strong barrier, stuck to the walls of the test tube, between the carbon tetrachloride and the methanolic layers. This prevents the former from being poured off together with the alcoholic supernatant solution.

Both the hexane and the carbon tetrachloride extracts were tested for the presence of the 17-OHCS. They did not contain any, as measured by the Porter-Silber color reaction.

The inclusion of the purification step with barium acetate is based on the fact that barium ions precipitate some of the polypeptidic compounds which produce color with sulfuric acid reagent. The efficiency of this precipitation is enhanced by addition of ethyl alcohol which decreases the solubility of these compounds. This step lowers the intensity of "background" color produced with sulfuric acid by 25 to 55 per cent.

The complete evaporation to dryness of the aqueous alcoholic extract is important. It was seen that if traces of water remain in the residue they either retain some interfering substances (disappearing on complete evaporation) or they possibly permit formation of a hydrated acetate compound with the pigment material of the extract, which may considerably increase the "background" color with the Porter-Silber reagent.

The last two steps are not necessary if the residue is hydrolyzed with β-glucuronidase.

The dry plasma residues obtained in the course of the experiments on purification described above were quadruplicated: one pair of them was extracted with the butanol-chloroform mixture, the other was hydrolyzed with β-glucuronidase and extracted with chloroform. In all instances the levels of the "total conjugates" were higher or, occasionally, equal to those of the glucuronides.

The precipitated proteins from 5 plasma samples were combined and re-extracted with ethyl alcohol (10 times the original volume of plasma). The alcohol extract was evaporated to dryness and the dry residue redissolved in water. Then subsequently extracted for free and conjugated 17-OHCS. None could be detected.

The reproducibility of the method expressed by coefficients of variation calculated from series of duplicate and quadruplicate estimations is shown in Table II.

Recovery experiments were performed with free and conjugated 17-OHCS. Pure hydrocortisone (free alcohol) was used for the "free" and a purified concentrated butanol extract of urine, prepared as described in the method

for urinary 17-OHCS,[10] was used for the "conjugated." The recovery rates were 94 to 100 per cent and 86 to 98 per cent for free and conjugated 17-OHCS, respectively.

TABLE II. REPRODUCIBILITY OF THE METHODS

METHOD	NUMBER OF DUPLICATES	NUMBER OF QUADRUPLICATES	RANGE (μg/100 ML.)	MEAN (μg/100 ML.)	COEFF. OF VARIATION
Free 17-OHCS	28		4 - 94*	35.2	2.3 %
Free 17-OHCS		10	18 - 67*	33.3	3.0 %
Conj. 17-OHCS	10		5 - 80*	29.6	5.9 %
Conj. 17-OHCS		6	12 - 40*	24.4	5.0 %

*High concentrations obtained after ACTH gel I.M. injection.

β-Glucuronidase Hydrolysis.—A series of experiments designed to determine the optimal conditions for hydrolysis of 17-OHCS glucuronides, using beef liver β-glucuronidase (Ketodase) established the quantity of the reagents and the duration and temperature of the incubation as given in the description of the method.

Table III shows a comparison of the results obtained with β-glucuronidase as applied to: (1) plasma buffered to pH 4.5 (previously extracted with chloroform); (2) plasma deproteinized with 2.5 volumes of absolute ethyl alcohol, evaporated to dryness, redissolved in distilled water (the volume equal to the original volume of plasma), buffered, and hydrolyzed; (3) plasma deproteinized with ethyl alcohol (to give final alcohol strength of about 70 per cent), acidified (acetate buffer, pH 4.5), and washed with hexane before evaporation to dryness; (4) and (5) the same as (2) and (3), respectively, but using methyl alcohol instead of ethanol. In all instances the hydrolyzate was extracted with 5 volumes of chloroform and subsequently treated as in the method described for "free 17-OHCS." The concentration of the 17-OHCS glucuronides found when methyl alcohol was used for the precipitation of the proteins and the hexane partition stage was included in the procedure (5) is given in the table as 100 per cent.

It is evident that if column chromatography is to be obviated in the purification of the extract of the hydrolyzate, the procedure described in this paper should be adopted.

TABLE III. HYDROLYSIS WITH β-GLUCURONIDASE APPLIED TO PLASMA WITH AND WITHOUT VARIOUS STEPS OF PRELIMINARY PURIFICATION

NO. OF EXPERIMENT*	ALCOHOL USED FOR DEPROTEINIZATION	HEXANE PARTITION STAGE	% OF 17-OHCS GLUCURONIDES CONCENTRATION FOUND
1	None	No	38
2	Ethanol	No	0 †
3	Ethanol	Yes	70 ‡
4	Methanol	No	65
5	Methanol	Yes	100

*See text.
†Reading with sulfuric acid reagent exceeds that with phenylhydrazine.
‡Very high reading with sulfuric acid reagent (1.090 at 410 mμ).

CLINICAL RESULTS

Normal Levels.—In 18 individuals, ranging in age from 22 to 60 years, values for the free 17-OHCS were found between 7.5 and 210 μg per 100 ml. of plasma (mean 12.4 μg); the conjugated 17-OHCS ranged from 6.0 to 24.0 μg per 100 ml. of plasma (mean 14.6 μg). In all instances blood was drawn between 9:30 and 10:30 A.M. to avoid possible differences due to diurnal variations.

In 8 normal subjects adrenals were stimulated by a single intramuscular injection of 120 I.U. of ACTH gel (at midnight) and the blood was drawn 9 hours later. The following results were obtained: free 17-OHCS range 33.0 to 65.0 μg per 100 ml. of plasma (mean 49.5 μg); conjugated 17-OHCS range 26.0 to 100.0 μg per 100 ml. of plasma (mean 64.9 μg).

DISCUSSION

The importance of the assessment of adrenocortical function by direct estimation of the levels of circulating corticosteroids in plasma has been widely recognized during recent years.[2, 12, 13]

Since the description in 1950 by Porter and Silber[11] of a relatively specific color reaction for 17,21-dihydroxy-20-ketosteroids (production of color with phenylhydrazine in strong sulfuric acid), various authors have developed methods based on this reaction. Nelson and Samuels[2] introduced column chromatography to the procedure; Bayliss and Steinbeck[3] modified and improved the latter; Bondy and Altrock[14] used an extensive solvent partition to purify the extract prior to the application of the color reaction; Silber and Porter[1] purified the chloroform extracts with sodium hydroxide solution and re-extracted the steroids from chloroform by means of an alcohol-acid reagent; Silber and Busch[4] increased the sensitivity of the latter method by purifying plasma with fat solvents prior to the actual extraction and modifying the quantities of the reagents.

All these methods are concerned with the free (unconjugated) 17-OHCS. Strangely enough, a very large fraction of the conjugated steroids has been generally neglected. Bongiovanni and associates[8, 9] developed a method based on the application of β-glucuronidase hydrolysis to deproteinized plasma with subsequent estimation of the liberated steroids by the method of Nelson and Samuels.[2] Reddy and co-workers[7] extended to plasma the method for total 17-OHCS in urine[5] by the introduction of additional steps (deproteinization, evaporation to dryness of the extracts) to the procedure. However, these two methods have not been widely adopted for clinical use.

The methods described in this paper allow a quick estimation of both free and conjugated 17-OHCS on the same sample of plasma. The method for free steroids is applicable to urine. The method for conjugated steroids is based principally on the extraction procedure used for the conjugates in urine.[10] This makes the methods for plasma and urine directly comparable—on one hand reducing the number of blanks and standards, on the other, facilitating such investigations as renal clearance or determination of the ratio of free to conjugated 17-OHCS.

SUMMARY

Methods have been developed for the estimation of free and conjugated 17-hydroxycorticosteroids in plasma.

The free steroids are extracted with chloroform and stabilized by addition of n-butanol to the extract to prevent their destruction during subsequent purification with anhydrous sodium carbonate. The steroid-alcoholic phase is then re-extracted by sulfuric acid and sulfuric acid-phenylhydrazine reagents, respectively.

The conjugated steroids are extracted with the n-butanol-chloroform mixture (the same as applied to urine[10]) after previous deproteinization of plasma with methyl alcohol and distribution of the supernatant between 70 per cent methanol and carbon tetrachloride and hexane, respectively. The steroid-alcoholic phase is re-extracted (from the butanol-chloroform extract) in the same way as in the method for free steroids.

Both methods are rapid, simple in performance, and suitable for routine clinical investigation. Their reproducibility expressed by the coefficient of variation is 2.3 and 5.9 per cent, respectively, for free and conjugated 17-hydroxycorticosteroids.

I wish to express my gratitiude to Professor W. Melville Arnott for providing the necessary funds and facilities for this work in his Department. I am thankful to Dr. A. C. Crooke for his supervision and to Dr. W. R. Butt, Chief Chemist to the Department of Clinical Endocrinology, for his help and advice; to Dr. F. C. Neale, Biochemistry Department, for his constant readiness to assist in the technical problems; and to Mr. Garfield Thomas, Chief Biochemist, Q.E.H., for the kind permission to use some of the apparatus of his Department in a part of this work. Finally, I wish to thank Professor G. F. Marrian for his valuable suggestions.

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A comparison is made between six generally used substrates for their relative specificity to prostatic and erythrocytic acid phosphatase. Results showed that a new substrate, alpha-naphthyl phosphate, was twice as specific as beta-glycerophosphate and 40-100 times as specific as all other substrates investigated. Acid phosphatase assays in serum should be specific for that enzyme arising from cancerous prostatic tissue. The substrate alpha-naphthyl phosphate* is specific for this enzyme.

Babson, A. L., and Read, P. A.: A new assay for prostatic acid phosphatase in serum. Am. J. Clin. Path. 32:1, 88-91, July 1959.

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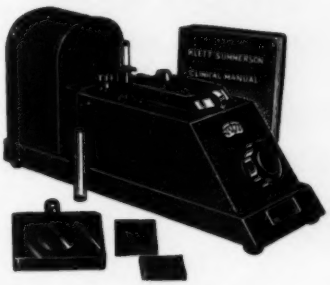

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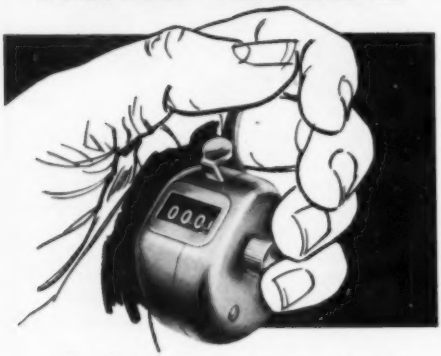

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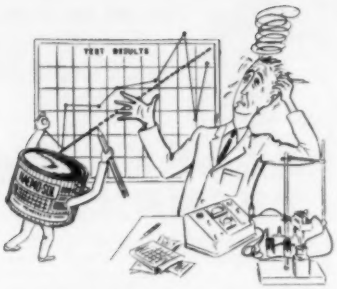

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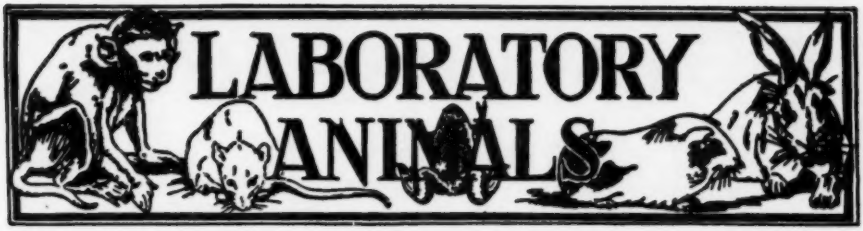

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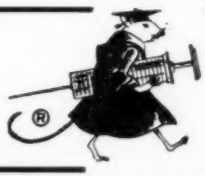

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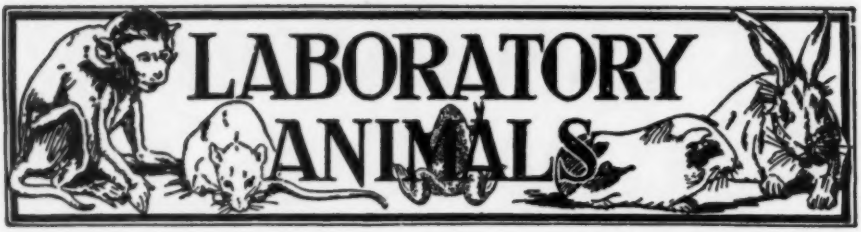

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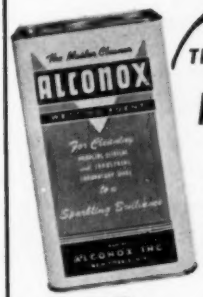

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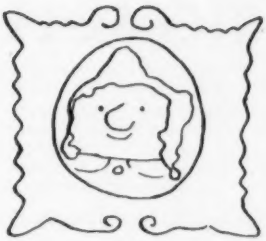

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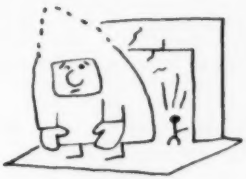

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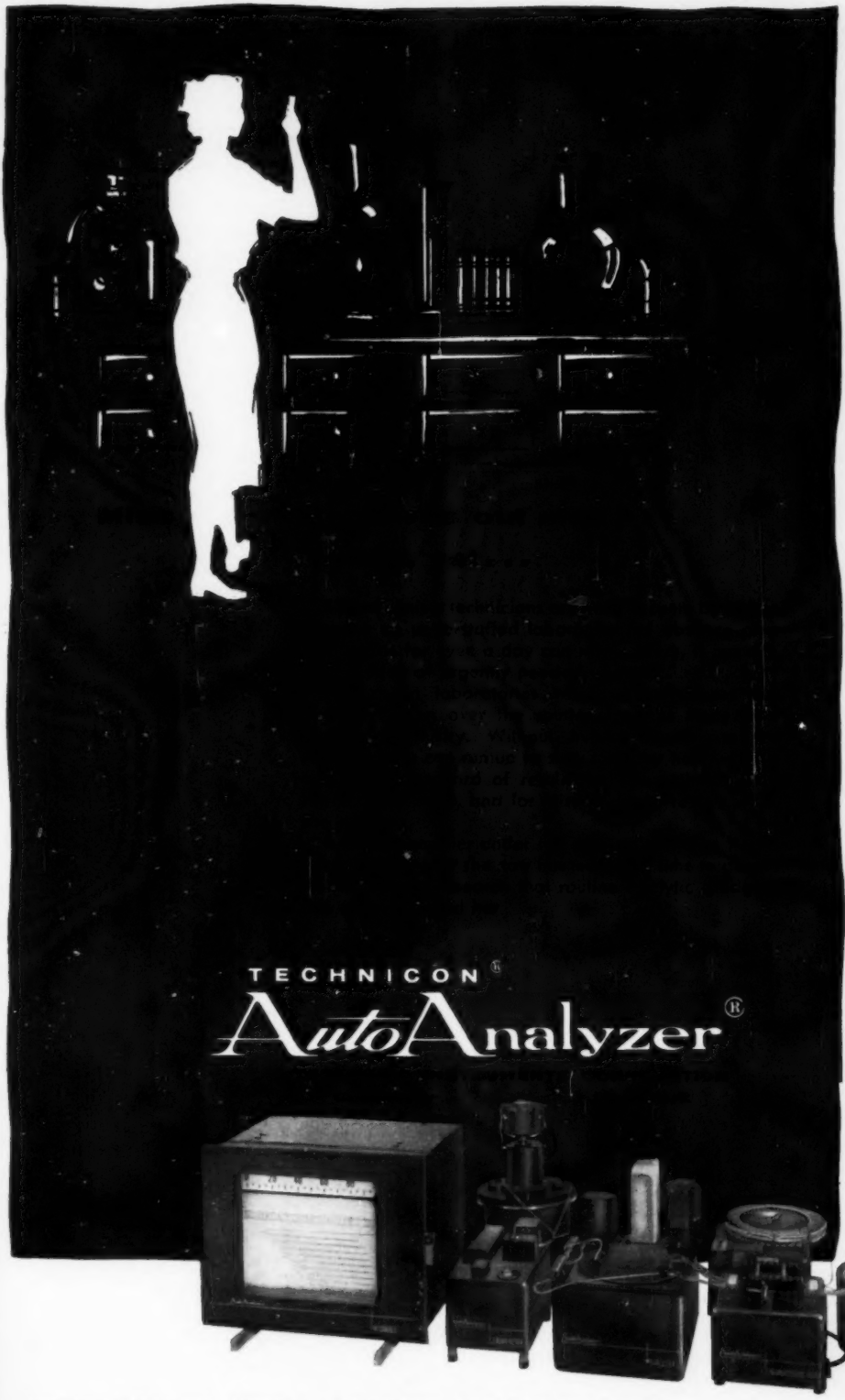

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